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(54) Title: ENHANCED STIMULATION OF ERYTHROPOIESIS

(57) Abstract

The present invention is directed to compositions comprising heme-containing components and to methods that stimulate erythropoiesis comprising administration of a composition of the invention, such as purified hemoglobin. Surprisingly, heme-containing components such as hemoglobin can induce erythopoiesis in the presence of decreased concentrations of erythropoietin (Epo) and, in fact, function synergistically with Epo. Further, heme-containing components can be used alone or in conjunction with Epo for the treatment of anemias and other disorders due to decreases in erythropoietin or iron. It has been discovered that, in the presence of Epo, both hemoglobin and cross-linked hemoglobin can compensate for the reduction in erythroid cell growth and differentiation that occurs in the presence of reduced concentrations of Epo. The effect is specific to erythropoiesis, as evidenced by a lack of growth of non-erythroid progenitors such as CFU-GM.

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ENHANCED STIMULATION OF ERYTHROPOIESIS

Reference to Related Applications

This application is a continuation of United States Provisional Patent Application No. 60/024,632, entitled Enhanced Stimulation of Erythropoiesis filed August 27, 1996.

Field of the Invention

The present invention relates to a novel method of stimulating erythropoiesis in mammals through the administration of a suitable amount of hemoglobin *in vivo* under conditions of decreased erythropoietin or iron levels.

Background of the Invention

Stimulation of Erythroid Progenitors by Erythropoietin

Erythropoiesis is an essential process required to replace worn out red blood cells that are continuously removed from the circulation. Some 200 billion red blood cells, having an average life span of 120 days, are produced daily in adults. Under normal physiological conditions, erythropoiesis is principally regulated by erythropoietin (Epo), a hormone produced by the kidney in response to hypoxia. Erythropoietin, produced by the renal peritubular endothelium, circulates to the bone marrow where it stimulates committed stem cell progeny called erythroid progenitors to produce red blood cells (Krantz, Blood 77:419-34, 1991; Roberts and Smith, J. Mol. Endocrin. 12:131-48, 1994). Including platelets and white blood cells, the total daily blood cell production amounts to half a trillion cells. This level of cell replacement constitutes only the steady state condition and reflects the remarkable endogenous proliferative capacity of stem cells.

Two kinds of functionally distinct erythroid progenitors have been identified based on their abilities to form morphologically recognizable colonies when grown in semi-solid media such as methyl cellulose. The first, the burst forming unit-erythroid (BFU-E), represents the most primitive erythroid progenitor and forms large multi-clustered hemoglobinized colonies. The second, the colony forming unit-erythroid (CFU-E), is a more

differentiated erythroid progenitor which forms smaller hemoglobinized colonies. The BFU-E is the earliest identifiable progenitor fully committed to erythropoiesis and has a larger capacity for self-renewal than the more mature CFU-E. Most BFU-E are quiescent with only 10 - 20% of the cells cycling at a given time, whereas, the majority of CFU-E are actively dividing. As BFU-E differentiate into CFU-E there is a loss in the expression of the primitive stem cell surface glycoprotein CD34, and an increase in the expression of receptors for erythropoietin and the iron transporter, transferrin. Although BFU-E express low numbers of receptors for erythropoietin, they are stimulated by Epo to proliferate and differentiate into CFU-E which, in turn, express higher levels of the Epo receptors. Erythroid differentiation beyond the CFU-E stage is dependent upon erythropoietin, and is characterized by the expression of the red blood cell membrane protein glycophorin A, the accumulation of additional erythroid-specific membrane proteins and the induction of hemoglobin synthesis.

Role of Erythropoietin in Erythroid Cell Proliferation and Differentiation

Erythropoietin stimulates erythroid proliferation and differentiation by interacting with a specific receptor expressed almost exclusively on erythroid progenitors. The Epo receptor is a member of the cytokine receptor superfamily and possesses the characteristic pentapeptide WSXWS motif (trp-ser-x-trp-ser; SEQ ID NO 1), along with four conserved cysteine residues within the extracellular domain (Krantz, Blood 77:419, 1991; Roberts and Smith, J. Mol. Endocrin. 12:131-48, 1994). Other members of the cytokine receptor superfamily include receptors for interleukin 2 (IL-2; β- and γ-chains), IL-3, IL-4, IL-6, IL-7, granulocyte-macrophage colony stimulating factor (GM-CSF), growth hormone and prolactin. All of these receptors have a similar predicted tertiary extracellular structure. The binding of erythropoietin to the Epo receptor results in the phosphorylation of the intracellular tyrosine kinase, JAK2, which, in turn, phosphorylates several intracellular proteins including STAT5, PI3 kinase, *vav* and others (Ihle, Nature 377:591-94, 1995). Evidence suggests that activation of these second messengers, and others, by phosphorylation

contributes to the Epo-induced proliferative response; however, the molecular basis which determines whether an erythroid cell will either proliferate or differentiate in response to Epo is unknown.

The later stages of erythroid differentiation are best characterized by the accumulation of the major red blood cell protein, hemoglobin, a tetrameric molecule consisting of an oxygen-binding heme moiety bound to each of four separate globin chains. At a concentration of ~28 pg/cell, hemoglobin is the most abundant protein present in the mature red blood cell, accounting for 95% of the cell protein. The high rate of red blood cell production in the marrow requires that red blood cell precursors synthesize 400 trillion molecules of hemoglobin every second. Erythropoietin-stimulated hemoglobin synthesis is coordinated within differentiating red cell precursors so that the synthesis of the constituent alpha and beta globin chains is concurrent with that of heme. Globin genes and genes encoding multiple enzymes along the heme-synthesis pathway (Weiss and Orkin, Expcr. Hematol. 23:99-107, 1995) are transactivated by the major erythroid transcription factor, GATA-1, which is expressed following the activation of the Epo receptor by the binding of Epo (Chiba et al., Nuc. Acid Res. 19:3843-48, 1991; Dalyot et al., Nuc. Acid Res. 21:4031-37, 1993; Busfield et al., Eur. J. Biochem. 230:475-80, 1995). Whether Epo will support primarily erythroid differentiation or proliferation appears to depend on the concentration of Epo and the status of the cell cycle. Low concentrations of Epo support β-globin production and prolong the G1 phase of the cell cycle, whereas higher Epo concentrations promote cell proliferation and shorten the G1 phase (Carroll et al., Proc. Natl. Acad. Sci. USA 92:2869-73, 1995).

Current Treatments of Anemia

Anemia is the pathological consequence of insufficient hemoglobin levels to meet the oxygen transport requirements of the body. There are several causes of anemia which include excessive blood loss, increased red blood cell destruction, decreased red blood cell production or hemoglobin synthesis, and abnormal hemoglobin production. Decreased

red blood cell production may result from inadequate iron incorporation (either iron deficiency or failure of iron mobilization, as seen in anemia of chronic disease), insufficient Epo production or bone marrow failure. Since the erythropoietic activity of bone marrow is intact in iron and Epo-dependent anemias, such anemias are treatable by iron or Epo administration, respectively.

Iron deficiency remains the most common cause of anemia both in the U.S. and worldwide. Deficiency may result from dietary insufficiency, blood loss or impaired iron absorption from the gastrointestinal tract. Anemia due to iron-deficiencies is typically treated by oral or intravenous iron administration. The effectiveness of oral iron treatment is limited by malabsorption, gastrointestinal side effects and noncompliance by the patient. Intravenous administration of iron does not suffer from these limitations, but the toxicity of iron dextran is often a problem (Fishbane et al., Am. J. Kid. Dis. 26:41-46, 1995). The most common adverse effects are pain and swelling at the injection site, arthralgia and fever. In addition, iron injections stain the skin brown. A more serious complication; however, is anaphylactic shock which may occur during or immediately following injection, and can be fatal if untreated. For this reason, a test for hypersensitivity is required before a course of iron dextran therapy is begun. Finally, there is the potential for iron overload with parenteral iron therapy. This can lead to hemochromatosis, and routine monitoring is recommended to ensure that this condition does not occur. In light of these difficulties, new iron delivery agents could have widespread clinical applicability.

In some patients, the body iron stores are intact, but hypoferremia develops because of disturbed iron metabolism. These are the hallmarks of anemia of chronic disease (ACD), which is associated with chronic infection, inflammatory diseases, trauma and neoplastic diseases. ACD is a very common and often clinically important condition. The etiology of this anemia is not completely understood, but many contributing factors have been identified (Means and Krantz, Blood 80:1639, 1992). The major problem appears to be impaired flow of iron from the tissues to the plasma. In the presence of inflammatory

mediators, such as IL-1, the macrophages responsible for normal hemolysis of senescent RBCs sequester the iron. As a result, iron is not released into the plasma for recycling and there in an insufficient supply of iron for erythropoiesis. A further complication of ACD is the fact that Epo levels do not rise appropriately for the degree of anemia. At present, treatment is aimed at resolving the underlying inflammation or infection. It would be useful; however, to develop new agents that could deliver iron directly to erythroid progenitors, while stimulating erythropoiesis in the presence of a blunted Epo response.

A variety of different disorders result in Epo-insufficiency, but the most classic examples are the diseases of the kidney. Patients with chronic renal failure typically exhibit Epo-dependent anemia due to the inability of their damaged kidneys to produce Epo. These patients require frequent dialysis to replace kidney function, and 90% of patients are clinically anemic. Until recently, the treatment of anemia in dialysis patients was via multiple transfusions. With the advent of recombinant human Epo, transfusions have been largely replaced by the administration of Epo. Indeed, ~88% of all dialysis patients are treated with Epo. Dialysis patients typically respond to Epo therapy with increases in reticulocyte count, hemoglobin level and hematocrit (Dunn and Markham, Drugs 51:299-318, 1996). Epo therapy is not without its drawbacks. One third of patients develop hypertension, which can generally be corrected using anti-hypertensive drugs. Iron deficiencies can also develop due to the increased transfer of iron from stores within the bone marrow to the rapidly proliferating erythroid progenitors for use in hemoglobin synthesis. The effectiveness of Epo therapy is reduced by insufficient iron and, thus, iron must generally be administered in conjunction with Epo for long-term therapy.

Erythropoietin is also approved for the treatment of anemia caused by chronic renal insufficiency, cancer or cancer therapy, as well as in patients infected with human immunodeficiency virus (HIV) who are undergoing zidovudine therapy. As the clinical applications of recombinant human erythropoietin expand, the cost of long-term therapy becomes a major concern. Typical Epo doses for dialysis patients are 225 Units/kg/week,

administered in three doses. Medicare reimbursement for Epo treatment in the U.S. is presently $10.00 per 1,000 Units (Section 13566, Omnibus Budget Reconciliation Act of 1993). Thus, the typical cost for a 70 kg patient would be ~$8,000 yearly. In 1995, 175,000 patients were on dialysis in the U.S. resulting in a market excess of $883 million for this indication alone (Amgen, 1995; Annual Report). The cost of this therapy is projected to be ~$1.1 billion in 1996 (Dau Hoffman; 1996, Vector Securities International). Novel therapies which would reduce the requirement for Epo in the treatment of anemia would thus be of benefit to patients and the health care system. A recent study suggests that Epo requirements could be substantially (~46%) reduced through the administration of intravenous iron (Fishbane et al., Am. J. Kid. Dis. 26:41-46, 1995). However, tissue iron overload is of concern. The discovery of other agents capable of reducing Epo requirements for the treatment of Epo-dependent anemias without producing iron overload would be advantageous.

Early Studies with Hemoglobin

As early as the 1940s, an increase in both blood hemoglobin and hematocrit was observed in anemic patients which received multiple injections of unmodified human hemoglobin (Amberson et al., J. Appl. Physiol. 1:469-89, 1994). The increases observed in these patients could have been due to the hemoglobin itself or to the efficient delivery of iron contained therein. However, the high levels of dissociated αβ globin dimers in the initial crude hemoglobin preparations resulted in renal tubular obstruction and decreased glomerular filtration rates (Spence, *Clinical Practice of Transfusion Medicine*, 3rd Edition, chapter 44, 1996). These crude hemoglobin preparations were also contaminated with endotoxin, residual cellular stroma and lipid fragments, all of which could contribute to adverse side effects including vasoconstriction, complement activation and the generation of free radicals. As a result of their adverse side effects which included hypertension, shock, renal damage, anaphylaxis and sometimes death, early studies using crude hemoglobin preparations were

discontinued. A direct role for hemoglobin in the induction of erythropoiesis was never established.

Crosslinked Hemoglobin

More recently, relatively nontoxic highly purified crosslinked hemoglobins have been developed primarily for use as blood substitutes. The crosslinked hemoglobins are stabilized to prevent the dissociation of hemoglobin into αβ dimers and, thus, do not adversely affect the kidneys. Furthermore, the steps used in purifying hemoglobin for crosslinking have reduced the adverse side effects previously observed with the earlier crude hemoglobin preparations. In a recent study, the infusion of a blood substitute, crosslinked bovine hemoglobin, into patients suffering from aplastic anemia was observed to significantly increase the number of circulating reticulocytes in three days, and the level of blood hemoglobin in seven days (Feola et al., Surg. Gynecol. Obstet. 174:379-86, 1992). However, no assessment of the effect of hemoglobin on hematopoiesis could be determined. The concurrent use of antimalarial and antibiotic drugs clouded the study's results. Although the authors stated that there was an erythropoietic effect, no direct studies were performed. Accordingly, the conclusions drawn by the study's authors must be interpreted as generalization. In fact, an independent assessment of this study attributed the clinical observations to the increased iron provided by the hemoglobin and not to the stimulation of erythropoiesis (Spence, *Clinical Practice of Transfusion Medicine*, chapter 44, 1996).

In another recent study, the hematological effects of crosslinked bovine hemoglobin were measured on human subjects (Hughes et al., J. Lab Clin. Med. 126:444-51, 1995). All subjects underwent a partial phlebotomy to remove ~15% of their blood volume, followed by a 3:1 hemodilution with Ringer's lactate and an intravenous injection of up to 45 g of crosslinked hemoglobin or the control solution (Ringer's lactate). Serum iron levels peaked at 8 hours and paralleled changes in the plasma levels of crosslinked hemoglobin, whereas ferritin levels peaked at 48 hours post-infusion. These data are consistent with the release of iron from the hemoglobin after it had been metabolized by the reticuloendothelial

system. Serum Epo levels increased two- to six-fold after 24 hours in all groups, but to a greater extent in subjects that received hemoglobin. The elevated Epo levels were considered an indirect effect attributed to hypoxemia induced by the phlebotomy/hemodilution procedure itself. No significant difference was observed in the hemoglobin or reticulocyte levels in the control or hemoglobin treated groups. The observed increase in Epo levels was apparently insufficient to stimulate erythropoiesis. In a more recent report, 0.4 or 0.6 g/kg body weight of the same crosslinked bovine hemoglobin was administered post-operatively to patients undergoing elective surgery (Hughes et al., Crit. Care Med. 24:756-64, 1996). Both the hematocrit and the corrected absolute reticulocyte count increased in patients treated with hemoglobin. Again, the reported stimulation of erythropoiesis by hemoglobin could not be distinguished from the effects of hemoglobin-mediated iron delivery, or the stimulation of erythropoiesis through an alternate mechanism.

In vitro studies demonstrated that crosslinked hemoglobin may protect erythroid progenitors from the toxic effects of 3'-azido-3'-deoxythymidine (AZT) (Fowler et al., Toxicol. Letts. 85:55-62, 1996). AZT can significantly inhibit the proliferation of erythroid cells in cultures of human $CD34^+$ bone marrow cells. Low doses of crosslinked recombinant human hemoglobin (0.01 - 1 μM) did not increase the proliferation of the erythroid cultures; however, when combined with AZT, the crosslinked hemoglobin reversed its toxic effects. A direct interaction of AZT with hemoglobin can not be ruled out as contributing to the reduced toxicity of the AZT in this study. Thus, *in vivo* data which has suggested a role for hemoglobin in the stimulation of erythropoiesis has yet to be directly demonstrated *in vitro*.

Stimulation of Erythroid Cells by Heme

Hemin, the ferric chloride salt of heme, has been shown to promote erythroid progenitor proliferation and differentiation in a variety of *in vitro* assays. Heme is the end product of a tightly regulated multi-enzymatic pathway, part of which occurs within the mitochondria. Intracellularly, heme is the prosthetic group for hemoproteins which include

hemoglobin, catalase and the cytochromes. Heme is involved in the regulation of the intracellular synthesis of these proteins at various levels including gene transcription, mRNA translation, transport, assembly and/or protein turnover (Padmanaban et al., Trends Biochem. Sci. 14:492-96, 1992). Exogenously added hemin induces erythroid differentiation in a number of erythroleukemic cell lines resulting in hemoglobin production (Ross and Sautner, Cell 8:513-20, 1976; Rutherford et al., Nature 280:164-65, 1979; Dean et al., Science 212:459-61, 1981). The stimulation of hemoglobin production by hemin is due to increases in both globin gene transcription and globin mRNA stability (Ross and Sautner, Cell 8:513-20, 1976). Hemin treatment specifically increases embryonic and fetal globin production in human cell lines and primary human erythroid cells without affecting β-globin production (Rutherford et al., Nature 280:164-65, 1979; Fibach et al., Blood 85:2967-74, 1995). Although hemin alone can induce erythroid differentiation of erythroleukemic cell lines, hemin requires the addition of exogenous Epo to stimulate differentiation of primary cultures of erythroid cells (Fibach et al., Blood 85:2967-74, 1995).

Along with its effect on erythroid differentiation, hemin also exerts a proliferative effect on erythroid progenitors. The *in vivo* administration of hemin into mice results in increases in BFU-E within the marrow (Monette et al., Exp. Hematol. 12:782-87, 1984). BFU-E colonies that formed in response to hemin treatment were larger and appeared earlier in culture than those from untreated samples (Holden et al., Exp. Hematol. 11:953-60, 1983). *In vitro*, hemin (50 - 200 μM) stimulates a two-fold increase in murine erythroid colonies over those stimulated by 0.1 U/ml Epo alone (Porter et al., Exp. Hemat. 7:11-16, 1979). In this study, hemin also stimulated erythroid colony formation in the absence of added Epo. Thus, hemin, which can be directly incorporated into hemoglobin by erythroid cells, may also influence both the proliferation and differentiation of erythroid cells.

The effects of hemin could be explained by the delivery of only iron; however, equimolar concentrations of the iron salt ferric chloride are unable to stimulate erythroid progenitor proliferation and/or differentiation to the same extent as hemin. Thus, it is

unlikely that hemin stimulates erythroid cells simply through the delivery of iron. *In vivo* the likely source of hemin (or heme) is from the breakdown of hemoglobin. One potential mechanism for the erythropoietic activity of hemoglobin is through the delivery of heme following its dissociation from its globin carriers. Free heme could be released from hemoglobin prior to its uptake by erythroid progenitors or, alternatively, intact hemoglobin could be taken up by the cells prior to the release of heme intracellularly. Intracellular heme could then stimulate erythroid progenitor proliferation and differentiation as previously described for hemin. Whether such a pathway occurs *in vivo* is presently unclear from the literature. Generally hemoglobin, which is released from lysed red blood cells, is cleared efficiently from the circulation. Free hemoglobin is bound in the circulation by haptoglobin and this complex is transported to the liver where it is rapidly cleared by hepatocytes. If haptoglobin becomes saturated then unbound hemoglobin is oxidized leading to the dissociation of heme from the globin chains. Free heme is then bound by hemopexin and transported to the liver where the heme group is either degraded to bilirubin or incorporated into cytochrome P-450 (Otto et al., Crit. Rev. Microbiol. 18:217-33, 1992). In studies investigating the role of hemin in erythroid progenitor proliferation and differentiation, there are no indications or suggestions of the potential role of hemoglobin in erythropoiesis, either directly or through the provision of heme.

Summary of the Invention

The present invention overcomes the problems and disadvantages associated with current strategies and designs and provides new compositions and methods for the treatment of human disorders.

One embodiment of the invention is directed to compositions comprising a heme-containing component. Heme-containing components include heme, hematin, hemoglobin and modifications of these components, or may comprise substantially little to no hemoglobin or other protein. Compositions may further comprise Epo or a functional

fragment of Epo. The two components may be linked via covalent, non-covalent or other chemical modifications.

Another embodiment of the invention is directed to methods for the stimulation of erythropoiesis comprising administration of a heme-containing composition to erythroid cells. Erythropoiesis may involve the proliferation of erythroid stem cells, the proliferation of erythroid progenitor cells or the expression of hemoglobin such as adult and/or fetal hemoglobin. Also, stimulation is preferably specific for erythroid cells and not for non-erythroid cells such as CFU-GM cells. Stimulation may involve differentiation of erythroid cells such as erythroid stem cells or erythroid progenitor cells.

Another embodiment of the invention is directed to methods for the stimulation of erythropoiesis in the presence of reduced amounts of endogenous Epo comprising administering a heme-containing composition to erythroid cells. Endogenous Epo concentration can be reduced in certain disorders. It has been discovered that a heme-containing composition and reduced amounts of Epo can function to stimulate and specifically stimulate erythropoiesis.

Another embodiment of the invention is directed to methods for alleviating one or more symptoms associated with anemia comprising administering a heme-containing composition to a patient. Preferably, the composition is substantially free of hemoglobin protein and the patient has a reduced endogenous level of Epo.

Another embodiment of the invention is directed to methods for providing usable iron or heme to iron-deficient or heme-deficient patients comprising administering a heme-containing composition to the patient. The heme-containing composition preferably contains heme or hematin in the substantial absence of hemoglobin protein. Patient treated may include patients suffering from porphyria such as acute hepatic porphyria.

Another embodiment of the invention is directed to methods for transplanting cells, and preferably stem cells or progenitor cells obtained from bone marrow, cord blood, leukophoresis or peripheral adult blood, comprising administering a heme-containing

composition to the cells. Compositions may be administered *in vivo* or *in vitro* to cells. Methods may also enhance successful engraftment processes of red blood cells.

Another embodiment of the invention is directed to methods for reducing the toxicity of chemotherapeutic agents administered to patents such as cancer patients comprising administering a heme-containing composition to the patient. Patients that can be treated include immunosuppressed patients such as patients undergoing organ transplants, patients subjected to viral infection or patients suffering from acquired immunodeficiency syndrome.

Another embodiment of the invention is directed to methods hemodilution comprising administering a heme-containing composition to a patient in association with the hemodilution process. Patients may be further administered Epo compositions.

Other embodiments and advantages of the invention are set forth, in part, in the description which follows and, in part, will be obvious from this description or may be learned from the practice of the invention.

Description of the Drawings

Figure 1 Fold expansion of umbilical cord blood progenitors at ambient oxygen.

Figure 2 Fold expansion of umbilical cord blood progenitors at 5% oxygen.

Figure 3 Fold expansion of umbilical cord blood progenitors with 0.2 Units of Epo at ambient oxygen.

Figure 4 Fold expansion of umbilical cord blood progenitors with 0.2 Units of Epo at 5% oxygen.

Figure 5 Inhibition of succinylacetone (SA) toxicity to erythroid progenitors.

Figure 6 Representative erythroid colony formation with various additions.

Figure 7 Representative HPLC profiles of the analysis of hemoglobin production.

Figure 8 Representative 3-dimensional frequency distributions of erythroid progenitors induced to differentiate in liquid culture in (a) the absence or (b) the presence

of HAo.

Figure 9 Fold expansion of adult blood progenitors at ambient oxygen.

Figure 10 Fold expansion of adult blood progenitors at 5% oxygen.

Figure 11 Fold expansion of adult blood progenitors with 0.2 Units of Epo at ambient oxygen.

Figure 12 Fold expansion of adult blood progenitors with 0.2 Units of Epo at 5% oxygen.

Figure 13 Fold expansion of $CD34^+$ progenitors with 2 Units of Epo at 5% oxygen.

Figure 14 Evaluation of HEMOLINK™ stimulation of erythropoiesis in anemic male rats.

Figure 15 Effect of HEMOLINK™ on ganciclovir toxicity.

Description of the Invention

As embodied and broadly described herein, the present invention is directed to novel compositions of heme-containing components and to novel methods for the treatment of disorders comprising the administration of compositions comprising heme-containing components to patients.

Role of Hemoglobin in the Treatment of Anemia

According to the current literature, iron alone is typically sufficient to augment the Epo-dependent stimulation of erythropoiesis. This can be simply attributed to the obligate requirement for iron in the synthesis of hemoglobin in response to Epo-stimulation. Hemin appears to be better than iron at enhancing Epo-stimulated erythropoiesis, but it may act independently of both iron, which it also provides in the form of heme, and of Epo through the direct activation of genes involved in hemoglobin synthesis. Hemin is more efficiently taken up and utilized by erythroid progenitors than free iron which can only be delivered to the progenitors via the receptor-mediated endocytosis of the specific plasma iron transporter, transferrin. Following the reduction of hemin by a ferri-reductase, the resultant

heme can be directly incorporated into nascent globin chains during hemoglobin assembly while free iron requires endogenous porphyrin synthesis before it could be incorporated into heme, and subsequently hemoglobin. Hemoglobin is a source of both heme and iron, but also enhances Epo-dependent erythropoiesis through mechanisms that are distinct from both Epo and iron or heme delivery.

It has been surprisingly discovered that hemoglobin can be used to directly and specifically stimulate primitive erythroid progenitors in the presence of low doses of Epo at which erythroid progenitor growth is otherwise severely limited. The exact mechanism of hemoglobin stimulation is substantially different from that of stimulation by hemin. Thus, not only does hemoglobin directly stimulate erythroid cells, hemoglobin also provides a readily available source of heme with all of its erythroid-specific stimulatory activity and which may be used in the synthesis of hemoglobin. In this regard, *in vivo* hemoglobin can act synergistically with Epo in the stimulation of erythropoiesis and consequently lower the amount of Epo required to generate a clinically beneficial erythropoietic response in anemic patients.

Accordingly, the present invention relates to compositions and method to specifically stimulate erythropoiesis in mammals through the administration of stabilized hemoglobin. Hemoglobin stimulates the proliferation and differentiation erythroid progenitors *in vitro*. More primitive erythroid progenitor cell, such as BFU-E cells, are more effectively stimulated by hemoglobin, but the more mature erythroid progenitor, CFU-E cells, are also significantly stimulated. Hemoglobin stimulation of erythropoiesis requires erythropoietin and does not stimulate non-erythroid cells. Most importantly hemoglobin synergizes with Epo to directly stimulate primitive multipotential progenitors and preferentially promotes their proliferation and differentiation into erythroid cells. Stimulation mediated by hemoglobin is not simply through the delivery or iron. Hemoglobin is more effective at treating iron-deficient anemia than equimolar iron. As disclosed herein, animal studies demonstrate that the stimulation of erythroid progenitors observed *in vitro* by

hemoglobin is matched by productive erythropoiesis *in vivo*. Hemoglobin is similar to hemin in the stimulation of erythroid proliferation, but is more effective than hemin in stimulating erythroid differentiation and results in significantly higher adult and fetal hemoglobin production. Thus, hemoglobin provides a stronger and more potent stimulus to developing erythroid cells than does heme which may be mediated by an independent mode of action. Nonetheless, part of hemoglobin's stimulatory activity resides in the effective delivery of heme which, in turn, stimulates erythroid cell proliferation and globin synthesis and which may be incorporated into hemoglobin. Thus, hemoglobin may be used to treat of anemias due to reduced erythropoietin levels, to lower the amount of erythropoietin administered to treat such anemias, to treat iron-deficient anemia, to treat anemias as a result of bone marrow failure or suppression and to treat other disorders in which heme delivery is important such as acute hepatic porphyria.

Heme-containing Compositions

One embodiment of the invention is directed to compositions comprising a heme-containing component. Heme-containing components include hemoglobin and heme. Hemoglobin as used herein includes, for example, natural or purified hemoglobin, recombinant hemoglobin, cross-linked hemoglobin such as, for example, those described in U.S. Patent Nos. 5,439,591; 5,545,328; and 5,532,352 (*e.g.* HEMOLINK™), hemoglobin fragments and chemically or genetically modified hemoglobin that, for example, prevent dissociation of the hemoglobin molecule or modify the oxygen-binding affinity, hemoglobin precursors, and hemoglobin in any oxidative state including the oxi and deoxy and met forms, nitric oxide (NO) and carbon monoxide (CO) forms and iron III (ferric) hemoglobin. Heme as used herein includes, for example, natural or purified heme or hemin including heme-arginate and heme-lysinate, heme-derivative such as, for example, those described in U.S. Patent No. 5,233,034, heme hydroxides, heme chloride, heme in neutral solutions, ferric heme (Fe^{+3}) or ferrous heme (Fe^{+2}), hematin such as lyophilized hematin, chemically or genetically modified forms of heme and heme fragments, heme precursors such as

protoporphyrin IX with or without iron, and heme in any oxidative state including the oxi and deoxy and met forms, nitric oxide (NO) and carbon monoxide (CO) forms and iron III (ferric) heme. Many of these forms of hemoglobin and heme are commercially available such as, for example, HEMOLINK™ (an O-raffinose cross-linked hemoglobin), NORMOSANG™ (heme arginate) and PANHEMATIN™ lyophilized hematin). These and additional forms of hemoglobin are disclosed in U.S. Patent Nos. 4,857,636; 5,189,146; 5,364,932; 5,399,671; 5,532,352; 5,250,665; and 5,334,707; as are methods for the cross-linking of hemoglobins and the conjugations of hemoglobin to other proteins as well as other chemical substances (*e.g.* nucleic acids, lipids, saccharides, amino acids, esters, alcohols, acids).

Preferably, compositions of the invention are pharmaceutical compositions that may contain one or more pharmaceutically acceptable carriers. Suitable pharmaceutically acceptable carriers include, for example, salts and salt solutions (*e.g.* Ringer's lactate), alcohols, water, glycerol, glycol such as polyethylene glycol, vitamins, minerals, proteins such as albumin, glycerin, oils, fatty acids, salts such as sodium, saccharides and polysaccharides, amino acids, starches, and combinations of these carriers. Pharmaceutical compositions can be administered directly to the patient or stored concentrated for dilution before use. Ready-to-use and concentrated forms may contain stabilizers and preservatives such as anti-oxidants and buffers that increase the stability of the heme-containing component and the composition.

Compositions of the invention are preferably physiologically stable and safe at therapeutically effective concentrations. Physiological stable compositions contain heme-containing components that do not break down or otherwise become ineffective upon introduction to a patient prior to having a desired effect. Components can also be made structurally resistant to catabolism, and thus, physiologically stable, by electrostatic or covalent coupling to specific reagents to increase physiological stability. Such reagents include salts and salt solutions (*e.g.* Ringer's lactate), amino acids such as arginine, glycine,

alanine, asparagine, glutamine, histidine or lysine, nucleic acids including nucleosides or nucleotides, or substituents such as carbohydrates, saccharides and polysaccharides, lipids, fatty acids, proteins, or protein fragments. Useful coupling partners include, for example, glycol such as polyethylene glycol, glucose, glycerol, glycerin and other related substances.

Compositions of the invention should also be safe at effective dosages. Safe compositions are compositions that are not substantially toxic, myelotoxic, mutagenic or teratogenic at required dosages, do not cause adverse reactions or side effects, and are well tolerated. Although side effects may occur, safe compositions are those wherein the benefits achieved from their use outweigh disadvantages attributable to adverse side effects. Unwanted side effects include nausea, vomiting, hepatic or renal damage or failure, hypersensitivity, allergic reactions, cardiovascular problems, gastrointestinal disturbances, seizures and other central nervous system difficulties, fever, bleeding or hemorrhaging, coagulation or thrombosis, serum abnormalities and respiratory difficulties.

Compositions of the invention preferably contain a heme-containing component in the substantial absence of protein. A number of studies have shown that hemoglobin protein can inhibit stem cell proliferation (WO 96/10634; based on U.S. Patent Applications Serial Nos. 08/316,424 and 08/535,882). Thus, the presence of protein, and in particular hemoglobin protein, can interfere with the desired biological effect. Substantial absence means that the amount of protein in the composition does not interfere with the positive effects of the heme-containing component. Typically, such compositions contain less than about 30% protein, less than about 20% protein, preferably less than about 5% protein, more preferably less than about 10% protein, and even more preferably less than about 1% protein. That absence of protein may represent the amount of hemoglobin protein or the amount of total protein in or added to the composition. For example, compositions of the invention that contain protein other than hemoglobin protein may contain protein that stabilizes the composition such as, for example, albumin or haptoglobin protein or protein fragments.

Hemoglobin and heme are typically obtained in large quantities from the blood of a mammal. Suitable mammals include primates, and preferably humans, and may also cattle, horses, sheep and swine. Blood may be obtained from specific areas or tissues such as peripheral blood obtained from an adult, child or infant (which can be used directly or proliferated *in vitro*, umbilical cord blood, blood obtained from bone marrow, discarded blood from blood banks and slaughter houses. Recombinant techniques have also successfully yielded expression of large quantities of heme-containing components from prokaryotic and eukaryotic cells. Eukaryotic cells which can express heme-containing products include animal or plant cells that have been genetically engineered or selected to express large amounts of hemoglobin protein, heme, hemin or 4 pyrrole nitrogens such as protoporphyrin IX, the precursor of heme. Large scale isolation and purification of heme-containing components can be performed by those of ordinary skill in the art using well-known and established procedures (*e.g.* U.S. Patent No. 5,439,591 and 5,545,328).

Another embodiment of the invention is directed to compositions comprising heme-containing components linked with an Epo-containing component. These two components can be linked via covalent or non-covalent means using procedures that are well-know to those of ordinary skill in the art. Covalent bonding can be performed, for example, by esterifying and thereby linking the heme-containing component or heme chemical moiety with the Epo component or chemical moiety. The chemical moiety is that portion of the entire component that is necessary for the functional effect. In the case of Epo, the Epo moiety may be a functional fragment of the Epo protein. With heme, the heme moiety may be that portion of heme or hemoglobin that synergistically functions with Epo. Coupling agents that are well-known in the art include dialdehydes such as glyoxal, malonic dialdehyde, succinic dialdehyde, glutaraldehyde, adipaldehyde, phthalic dialdehyde, terephthaldehyde, 3-methylglutaraldehyde, and propyladipaldehyde, and malonic dialdehyde, O-raffinose and diaspirin, all of which are commercially available (*e.g.* U.S. Patent Nos. 3,980,764; 4,001,200; 4,001,401; 4,053,590; 4,136,093; 4,291,013; 4,336,248; 4,497,932;

4,540,564; 4,842,856; 4,849,141, 4,868,158; and 4,879,272). In addition, coupling techniques are well-known to those of ordinary skill in the art (*e.g.* Hermanson, *BioConjugate Technology* Academic Press, San Diego, CA, 1996; Wong *Chemistry of Protein Conjugation and Cross Linking* CRC Press, Inc., 1991).

Coupling may be through covalent or non-covalent interaction. Covalent bonding can be performed using peptide bonding of one or more amino acids of Epo with the heme-containing component. Non-covalent bonding can be performed using hydrogen bonding, hydrophobic interactions and/or subunit interaction by attaching to one component a coupling agent such as, for example, an adhesion molecule, a nucleic acid, a biotin or a biotin derivative, and attaching to the other component a complementary coupling agent such as, for example, an adhesion molecule, a nucleic acid, avidin, streptavidin or a streptavidin derivative. Other coupling agents that can be utilized are well-known to those of ordinary skill in the art. The interactions achieved in this manner are specific and sufficiently stable to maintain the resulting structure *in vitro* and *in vivo*. Linking may also be achieved using coupling agents and spacers that, preferably, do not interfere with the biological effect of the combination.

Compositions comprising a heme-containing component linked with an Epo-containing component, may further comprise the addition of excess heme-containing components, or Epo or Epo-containing component to obtain a ratio of Epo activity to Heme activity necessary to achieve the desired result such as, for example, the alleviation of one or more symptoms associated with a disorder. Such compositions are preferably useful for the treatment of patients who are Epo deficient, but are unable to tolerate or respond to direct stimulation with Epo. Further, as these two moieties act synergistically, as disclosed herein, compositions containing both components have wide utility. Alternatively, compositions may comprise a heme-containing component and an Epo component as a heme carrier.

Many of the experiments disclosed herein were conducted using adult human hemoglobin obtained from red blood cells and purified by displacement chromatography

processes as described in U.S. Patents 5,439,591 and 5,545,328. This purified, but uncross linked product is referred to as HAo. Cross-linked hemoglobin used included the O-raffinose modified form of hemoglobin referred to as HEMOLINK™. While administration can be with either uncross linked human hemoglobin or stabilized crosslinked human hemoglobin, crosslinked, stabilized hemoglobin is preferred for *in vivo* administration to humans. The ability of cross-linked hemoglobin to stimulate erythropoiesis provides potential therapeutic applications for the use of hemoglobin in the treatment of anemia and other disorders. In most situations, unmodified hemoglobin cannot be used in humans as the tetrameric molecule readily dissociates into its substituent αß dimers. These dimers are rapidly cleared from the bloodstream by the kidney which can result in renal damage. In contrast, hemoglobin can be modified to prevent tetramer dissociation via chemical or genetic approaches. Hemoglobins modified to prevent tetramer dissociation are not readily cleared by the kidney and consequently do not cause renal damage. HEMOLINK™, an example of a chemically crosslinked hemoglobin, is prepared by treating human hemoglobin with a polyaldehyde (O-raffinose) obtained by oxidatively ring-opening the saccharide raffinose, which crosslinks the hemoglobin tetramer across the 2,3-diphosphoglycerate binding site. Other crosslinking agents may be used to stabilize the hemoglobin tetramer, and such agents are well known in the art and described in the patent literature. HEMOLINK™ is discussed and used by way of example only.

Stimulation of Erythropoiesis

Another embodiment of the invention is directed to methods for the stimulation of erythropoiesis. Methods comprise the administration of a therapeutically-effective amount of a heme-containing composition of the invention. An effective or a therapeutically effective amount is that amount of the composition or the heme-containing component of the composition that is effective at detectably stimulating erythropoiesis of cells, preferably erythroid cells, or cells of a patient. Erythropoiesis is detectable if, for example, stimulation can be observed in culture or stimulation overcomes one or more patient symptoms

associated with lack of erythropoiesis. A therapeutical effective amount is relation to a disorder is that amount which has a beneficial effect to the patient by alleviating one or more symptoms of the disorder or simply reducing premature mortality. For example, a beneficial effect may be a decrease in pain, a decrease in duration, frequency or intensity of a symptom, an increased hemocrit, an improved erythropoiesis, an increased reticulocyte count, an increased red cell count, an increased total hemoglobin, an increased peripheral blood flow, a decreased hemolysis, decreased fatigue or an increased strength. Preferably, a therapeutic amount is that amount of the heme-containing component that stimulates or enhances the expression of hemoglobin such as, for example, adult or fetal hemoglobin globin, or the proliferation of fetal or adult hemoglobin expressing cells.

Patients that can be effectively treated by the compositions of the invention include patients that have a lower than normal level of Epo or less than normal ability to produce or express Epo. Surprisingly, heme-containing compositions of the invention work effectively in the presence of reduced amounts of Epo, unlike other treatments which require that high or at least normal levels of Epo be maintained in the patient. Reduced Epo concentration are concentrations that are less than the concentration found in otherwise normal patients or the average of otherwise normal patients. That amount of endogenous Epo in normal human adults is typically between about 10 to 20 mUnits of Epo per ml of peripheral blood. Accordingly, less than normal amounts are amounts that are less than about 10 mU/ml, such as less than about 5 mU/ml, preferably less than about 2 mU/ml, and more preferably less than about 1 mU/ml, or none.

In the treatment of many disorders including anemias such as ACD, Epo is administered to the patient in fairly large amounts. Target serum or blood plasma concentrations are desired to be greater than 1 U/ml, preferably greater than 2 U/ml, and more preferably greater than about 5 U/ml. Epo concentrations desired upon administration of a heme-containing component are less than about 5 U/ml, preferably less than about 2

U/ml, and more preferably less than about 1 U/ml, and even more preferably less than about 0.2 U/ml.

Another embodiment of the invention is the co-administration of both a heme-containing component and Epo or a functional fragment or derivative of the Epo protein. However, as the heme-containing component acts synergistically with Epo, the amount of Epo required for the same result is substantially reduced. This results in a substantial savings in time, cost and overall success of a treatment. For example, Epo treatments that can be performed using less Epo will reduce undesirable side effects that may be associated with Epo administration, can render the Epo that is administered more effective to the patient, and reduces or eliminates problems associated with tolerance to the composition, all without decreasing the rate of success. In addition, as doses can be reduced, overall care may include other treatments in combination with Epo therapy.

Compositions of the invention are preferably specific for the stimulation of erythropoiesis in erythroid progenitors such as CFU-GEMM cells, BFU-E cells and CFU-E cells. Compositions preferably do not significantly stimulate the proliferation or differentiation of non-erythroid cells such as CFU-Baso cells, CFU-Mast cells, CFU-GM cells, CFU-Eo (Eosinophil) cells, and lymphoid progenitor cells (CFU-L). A stimulation is not significant if the resulting effect is not either beneficial or harmful to the results of the treatment. The stimulation of erythropoiesis may include the stimulation of differentiation of erythroid cells, or the increased expression of hemoglobin such as adult or fetal hemoglobin. Surprisingly, stimulation can involve the stimulation of expression of both adult and fetal forms of hemoglobin.

Another embodiment of the invention is directed to the treatment of anemic patients to ameliorate one or more symptoms associated with the disorder. As the heme-containing composition acts synergistically with Epo, combination treatments of heme or hemoglobin plus Epo can be considered and will be more successful that either single treatment alone. Further, in many anemic disorders, patients have a fairly low endogenous

level of Epo which may be the cause, effect or simply a consequence of the anemia.

Disorders that can be treated by the methods of the invention include diseases and maladies that can be characterized as a direct or indirect consequence of a defect of hematopoiesis, a defect in the production or expression of hemoglobin, or a defect or deficiency in erythroid cell differentiation and development. Such disorders include, for example, anemias such as sickle cell anemia, hemolytic anemia, infectious anemia, aplastic anemias, hypoproliferative or hypoplastic anemias, sideroblastic anemias, myelophthisic anemias, antibody-mediated anemias, anemias due to enzyme-deficiencies or chronic diseases, anemias due to blood loss, radiation therapy or chemotherapy, thalassemia including α-like and β-like thalassemia, or globin disorders due to infections of viral, bacterial or parasitic origin such as malaria, trypanosomiasis, human immunodeficiency virus and other retroviruses, a polyoma virus such as JC virus, a parvovirus, or a hepatitis virus such as human hepatitis viruses types A-G. Treatable disorders also include syndromes such as hemoglobin C, D and E disease, hemoglobin lepore disease, and HbH and HbS diseases.

Additional disorders that can be treated by the compositions of the invention include disorders associated with iron and heme deficiencies including porphyria such as acute hepatic porphyria. Heme containing components of the composition provide excellent vehicles for transferring iron or heme, in an acceptable form, into cells for the production of hemoglobin, cellular proteins and enzymes, such as all cytochromes including cytochrome p450, and other functions associated with cellular iron or heme. The administration of useable iron is also important in the treatment of disorders such as anemia of chronic disease (ACD), and in bone marrow transplants. Premature newborns often require stimulation of erythropoiesis with the concomitant addition of iron. However, newborn typically have very low levels of Epo and do not respond well to the direct administration of iron or iron infusion complexes such as INFUFER™. These patients can especially benefit with the administration of compositions of the invention which can provide heme-containing

components that function effectively with low concentrations of Epo and can involve co-administration of Epo and heme-containing components.

Treatment with compositions of the invention ameliorates one or more symptoms associated with a disorder. Symptoms typically associated with disorders associated with erythropoiesis include, for example, anemia, tissue hypoxia, organ dysfunction, porphyria, abnormal hematocrit values, ineffective erythropoiesis, abnormal reticulocyte (erythrocyte) count, abnormal iron load, the presence of ring sideroblasts, splenomegaly, hepatomegaly, impaired peripheral blood flow, dyspnea, increased hemolysis, jaundice, anemic crises and pain such as angina pectoris.

The patient may be a domesticated animal such as a dog, cat, horse, cow, steer, pig, sheep, goat or chicken, or a wild animal, but is preferably a primate such as a human. Administration may be to an adult, an adolescent, a child, a neonate, an infant or *in utero*. Another aspect of the invention is the treatment of newborns with compositions of the invention as newborns and other neonates, who typically have very low levels of Epo. Accordingly, treatment of newborns and newborns who may be suffering from hemoglobin deficiency would substantially benefit from the compositions of the invention.

Administration of the composition may be short term, continuous or sporadic as necessary. Patients with a suspected or diagnosed with a erythropoietic disorder may only require composition treatment for short periods of time or until symptoms have abated or have been effectively eliminated.

Preferably, patients that can benefit from the methods of the invention are patients undergoing cell transplantation such as, for example, stem cell transplantation by bone marrow replacement, cord blood transplantation, leukophoresis, mobilized adult peripheral blood. In, for example, mobilized adult peripheral blood transplantation, peripheral blood is obtained from the patient and treated with one or more cytokines to promote differentiation of proliferation of cells such as stem cells or progenitor cells. Treated cells are than mobilized or infused into the same or an immunogenically matched

patient after the patient was subjected to radiotherapy or chemotherapy. In this manner, transplanted cells, which may be an expanded population of the patient's own cells, can re-populate the otherwise cell depleted patient. Red blood cell engraftment can be enhanced as well as bone marrow replacement and cell enrichment following leukophoresis.

Patients undergoing chemotherapy or radiation therapy can also benefit from the compositions of the invention. Surprisingly, it has been discovered that compositions of the invention can reduce toxicity associated with other forms of therapy. For example, cancer patients being treated or about to be treated with chemotherapy can be co-administered a heme-containing composition of the invention. Cellular toxicity of the chemotherapeutic agent is substantially reduced by the heme-containing component. Substantially reduced means that toxicity is reduced such that treatment may continue, that side effects attributed to the treatment can be more easily tolerated by the patient, or that increased amounts of the chemotherapeutic agent can be utilized. Chemotherapeutic agents which show this affect include nucleoside analogs such as, for example, acyclovir (ACV), ganciclovir (GCV), famciclovir, foscarnet, ribavirin, zalcitabine (ddC), zidovudine or azidothymidine (AZT), stavudine (D4T), lamivudine (3TC), didanosine (ddI), cytarabine, dideoxyadenosine, edoxudine, floxuridine, idozuridine, inosine pranobex, 2'-deoxy-5-(methylamino)uridine, trifluridine and vidarabine. Examples of protease inhibitors that show this affect include saquinivir, ritonavir and indinavir. Other agents include the cyclophosphamide such as alkylating agents, the purine and pyrimidine analogs such as mercaptopurine, the vinca and vinca-like alkaloids, the etoposides or etoposide like drugs, the antibiotics such as deoxyrubocin and bleomycin, the corticosteroids, the mutagens such as the nitrosoureas, antimetabolites including methotrexate, the platinum based cytotoxic drugs, the hormonal antagonists such as antiinsulin and antiandrogen, the antiestrogens such as tamoxifen an other agents such as doxorubicin, L-asparaginase, DTIC, mAMSA, procarbazine, hexamethylmelamine and mitoxantrone.

Compositions can be directly or indirectly administered to the patient. Indirect administration is performed, for example, by administering the composition to cells *ex vivo* and subsequently introducing the treated cells to the patient. The cells may be obtained from the patient to be treated or from an immunologically matched or unmatched patient, or a genetically related or unrelated patient (*e.g.* syngeneic or allogeneic cells). Related patients offer some advantage by lowering the immunogenic response to the cells to be introduced. For example, using techniques of antigen matching, immunologically compatible donors can be identified and utilized. Administration for *in vivo* stimulation can be by any means that is safe and effective for the patient.

Direct administration of a composition may be by parenteral administration, or by pulmonary absorption such as sprays to nasal areas which can provide rapid access to the bloodstream. Parenteral administration may be by intravenous injection, intra-arterial injection or direct injection or other administration to one or more specific sites. Injectable forms of administration are sometimes preferred for maximal effect in, for example, bone marrow. Administration can be by bolus injection or sequential over time (episodically) such as every one, two, four, six or eight hours, or every day (QD), or every other day (QOD). When long term administration by injection is necessary, venous access devices such as medi-ports, in-dwelling catheters, or automatic pumping mechanisms are also preferred wherein direct and immediate access is provided to the arteries in and around the heart and other major organs and organ systems. Effective *in vitro* amounts are typically less than therapeutically effective *in vivo* amounts as *in vivo*, the component distributes throughout he body. However, concentrations in specific areas such as, for example, bone marrow, may be necessary to achieve therapeutically effective amounts.

Another embodiment of the invention is directed to enhancing the success of cell transplantation procedures and, preferably, transplantation of stem cells, progenitor cells and red blood cells such as, for example, in red blood cell engraftment processes. Stem cells and other types of cells for transplantation may be obtained from bone marrow, cord blood,

leukophoresis procedures, or peripheral blood collection. In blood collection, cells are obtained from, for example, adult patients and cultured *in vitro* in the presence of cytokines such as Epo, growth factors (*e.g.* fibroblast growth factor, stem cell growth factor), bone morphogenic proteins, interleukin (IL) such as IL-1, IL-2, IL-3, Il-4, IL-5, IL-6, IL-7, *etc.*, and preferably IL-3, that stimulate proliferation and/or differentiation of the cells. the patient is than subjected to therapy such as chemotherapy or radiotherapy that destroys one or more cell populations in the body. Cultured cells are than mobilized back into the patient to re-populate the one or more of the now cell depleted systems including organ systems. These methods can increase the success of red blood cell engraftment processes and other transplantation procedures. Methods are also useful when the patient is immunosuppressed such as in association with an infection, or by design subsequent to organ transplantation. Infections that can cause immunosuppression include viral infection such as, for example, infection of Epstein Barr virus, adenovirus, cytomegalovirus and other herpes viruses, and retroviruses including T cell and B cell viruses which can induce disorders associated with acquired immunodeficiency syndrome.

Another embodiment of the invention is directed to a method for hemodilution comprising administering a composition of the invention to a patient undergoing a hemodilution process. Hemodilution involves the extraction or removal of blood for a patient prior to a treatment therapy. Treatment may require subsequent re-population with the patient's blood cells or infusion of blood during treatment such as during surgery. Compositions of the invention can be administered before blood removal to maximize the hemoglobin or erythroid cell content of the blood, or after removal to maximize recovery of the patient hemoglobin or erythroid cell levels, or both before and after therapy.

The Detection of Erythroid Progenitors in the Colony Formation Assay

Hemoglobin enhances the growth of erythroid progenitors, notably the BFU-E progenitor population as shown by a well-known technique in the field of erythropoiesis research. This technique, referred to as the colony formation assay (CFA), is the most

widely used biological assay to identify and enumerate erythroid progenitors present in hematopoietic tissue such as blood and bone marrow. Cell populations containing erythroid progenitors are plated in semi-solid suspensions of methyl cellulose, agar, low melting agarose or related substances in nutrient culture medium containing 1 - 3 U/ml Epo and 10 ng/ml IL-3 to specifically stimulate erythroid progenitors. Plates are incubated at 37°C for 14 days during which time the erythroid progenitors form characteristic hemoglobinized (red) colonies. Two distinct, but related erythroid progenitors can be distinguished based on colony size and morphology: BFU-E, the most primitive recognizable erythroid progenitor forms large multilobular colonies whereas the more mature CFU-E forms smaller spherical colonies. Under these conditions other myeloid progenitors, notably the colony forming unit - granulocyte-macrophage (CFU-GM), also form morphologically identifiable non-hemoglobinized (white) colonies which are readily distinguished from erythroid colonies. Additional cytokines, notably IL-1, IL-6, stem cell factor, flt-3 ligand, and granulocyte-macrophage colony stimulating factor, may be included in the CFA to stimulate both erythroid and nonerythroid progenitor proliferation.

Hematopoietic Progenitors in Human Umbilical Cord and Adult Peripheral Blood

Umbilical cord blood is a rich source of hematopoietic progenitors. For this reason, umbilical cord blood is often used as a bone marrow replacement. The approximate progenitor numbers in cord blood and adult blood are shown Table 1. In adult peripheral blood, hematopoietic progenitors are present at reduced levels compared to cord blood.

TABLE 1

Hematopoietic Progenitor Number in Umbilical Cord and Adult Blood

Cells	Progenitor Number per 1 x 10^5 Cells								
	BFU-E			CFU-E			CFU-GM		
	Mean	S.D.	n	Mean	S.D.	n	Mean	S.D.	n
Umbilical Cord	237	185	95	214	180	95	41	35	93
Adult Blood	29	15	8	32	38	8	6	7	8

Hemoglobin Stimulates Erythroid Progenitor Proliferation

The addition of HAo to methyl cellulose at the initiation of a CFA increases the number of erythroid progenitor colonies obtained. The effect of HAo on erythroid progenitor growth is concentration-dependent; significant stimulation of erythroid progenitor colony formation is observed at 7.8 μM HAo (Table 4). The highest concentration of HAo tested, 15.6 μM (62 μM heme equivalents), produces the greatest increase in erythroid progenitors. Under these conditions there are approximately 3.2- and 1.8-fold increases in BFU-E and CFU-E, respectively, when compared to the colonies which form on control plates treated with only the HAo vehicle (lactated Ringers solution). This demonstrates that HAo can stimulate erythropoiesis and that the more primitive BFU-E progenitors are more responsive to HAo than the CFU-E. Thus, HAo is specific for promoting erythropoiesis and that it does not stimulate CFU-GM.

The ability of crosslinked hemoglobins such as HEMOLINK™ to enhance the growth of erythroid progenitors was tested in a CFA. HEMOLINK™ stimulates a 2.8-fold increase in BFU-E and a 1.4-fold increase in CFU-E, similar to hemoglobin. These data indicate that hemoglobin, modified to render it stable against tetramer dissociation, is equivalent to unmodified native hemoglobin in the stimulation of erythropoiesis. Thus, hemoglobin modified in any manner to prevent tetramer dissociation (via chemical or genetic approaches) can elicit a similar response by the erythroid progenitors.

Hemoglobin Stimulates Erythroid Progenitor Differentiation

The ability of purified adult hemoglobin to stimulate erythroid progenitor differentiation has also been studied using a liquid culture system containing Epo. In this liquid culture system hemin is known to accelerate erythroid differentiation of progenitors obtained from normal adult peripheral blood, and to selectively increase fetal hemoglobin production in these cells. Primary low density mononuclear cells (LDMNC) isolated from cord blood were tested in this culture system. Erythropoietin (2 U/ml) was found to stimulate erythroid differentiation of cord blood LDMNC in liquid culture resulting in an

increase in glycophorin A and CD71 co-expression as well as benzidine staining indicative of hemoglobin production. The addition of HAo (7.8 - 15.6 μM) to the cultures results in a further increase in glycophorin A/CD71 co-expression (80% vs. 53% in the Epo only control) and benzidine staining (82% vs. 43% in the Epo only control) after 20 days in culture. These data are consistent with a role for hemoglobin in the stimulation of erythroid differentiation in addition to the stimulation of erythroid progenitor proliferation.

Hemoglobin is Equivalent to Hemin in Stimulating Erythroid Progenitor Proliferation

To determine if hemoglobin elicits its effect on erythroid progenitors through the provision of its substituent iron and/or heme components, the effect of HAo was compared directly to $FeCl_3$ and hemin (the ferric chloride salt of heme). $FeCl_3$ (100 μM) stimulates a 2.1-fold increase in BFU-E number relative to untreated controls, however it does not significantly increase the number of CFU-E (see Table 4). Lower concentrations of $FeCl_3$ do not stimulate a significant increase in erythroid progenitor number. The concentration of $FeCl_3$ required to increase the number of erythroid progenitors is greater than that required for hemoglobin (7.8 μM; 31 μM iron equivalents). These data indicate that hemoglobin does not simply stimulate erythroid progenitors through the delivery of free iron. Hemin, on the other hand, stimulates an increase in erythroid progenitors that is similar to the increase observed in response to hemoglobin. Hemin (64 μM) stimulates a 2.3- and 1.8-fold increase in BFU-E and CFU-E numbers, respectively, and, at the most effective concentration tested (100 μM), stimulates a 2.9- and 2.0-fold increase in BFU-E and CFU-E colonies, respectively. Thus, hemoglobin is similar to hemin in its ability to enhance the growth of erythroid progenitors.

Hemoglobin is Better Than Hemin in Stimulating Erythroid Progenitor Differentiation

Succinylacetone (SA), a heme synthesis inhibitor, induces apoptosis in erythroid progenitors. This effect can be overcome by the addition of exogenous hemin but not $FeCl_3$ to the cells. To further demonstrate the mechanism by which HAo stimulates erythroid progenitor growth, the effect of hemoglobin on erythroid progenitor growth was

tested in the presence of SA. As expected, hemin (100 μM) is able to completely overcome the SA inhibition of erythroid colonies. HAo (15.6 μM; 62 μM heme-equivalents) is partially able to overcome the inhibitory effect of SA on erythroid colony formation, but it is unable to fully reverse this toxicity. HEMOLINK™ is similar to HAo in reducing SA toxicity while high concentrations of $FeCl_3$ (250 μM) are less effective than lower concentrations of hemoglobin. These data again indicate that hemoglobin may partly stimulate erythroid progenitor growth and globin synthesis through the delivery of heme to the cells, similar to hemin. The reduced effectiveness of hemoglobin in counteracting SA may be due to the slightly lower molar concentration of heme theoretically available (62 μM for HAo vs. 100 μM for hemin) in the administered hemoglobin, or to less efficient heme delivery or uptake of intact hemoglobin by the responding cells. However, the erythroid colonies which form in the presence of hemoglobin are usually larger and redder than those stimulated by higher molar concentrations of hemin indicating that hemoglobin has additional activity in the stimulation of erythropoiesis, and that it may also promote erythroid progenitor differentiation.

The more intense redness of the colonies stimulated by hemoglobin cannot be attributed to non-specific binding or uptake of hemoglobin by erythroid cells since non-hemoglobinized colonies on the same plate, such as CFU-GM, remain white in the presence of hemoglobin. Also, no red colonies are formed in plates containing hemoglobin but not Epo. The increased redness of erythroid colonies correlates with a higher intracellular hemoglobin content as determined by HPLC. Hemin stimulated cells are less red and contain lower levels of predominately fetal hemoglobin while the redder hemoglobin-stimulated cells synthesize overall higher levels of both adult and fetal hemoglobin. Thus, the mechanism by which hemoglobin enhances the growth of erythroid progenitors is distinct from that of hemin since hemoglobin cannot entirely reverse the toxicity of a heme synthesis inhibitor yet it is a stronger inducer of differentiation than hemin.

Low Oxygen Tensions Increase Erythroid Progenitor Proliferation

Normally colony formation assays are conducted at 37°C under 5% CO_2 at ambient oxygen tensions; however, it has been reported that erythroid progenitor colony formation is enhanced at lower, more physiological oxygen tensions, such as 5% O_2 (Lu and Broxmeyer, Exp. Hematol. 13:989-93, 1985; Maeda et al., Exp. Hematol. 14:930-34, 1986; Pennathur-Das and Levitt, Blood 69:899-907, 1987; Ono and Alter, Exp. Hematol. 23:1372-77, 1995; Weinberg et al., Hemoglobin 19:263-75, 1995). Ambient oxygen tensions (~20% O_2) correspond to ~160 mm Hg whereas 5% O_2 corresponds to ~ 40 mm Hg. The partial pressure of oxygen in arterial and venous blood is 100 and 40 mm Hg, respectively. It is likely that the partial pressure of oxygen within the bone marrow, where erythroid progenitors normally reside, is less than 40 mm Hg.

Higher oxygen tensions favor the formation of highly reactive oxygen species (ROS) that can induce cellular damage as a result of lipoperoxidation and the modification of other macromolecules such as nucleic acids and proteins. The generation of ROS in erythroid progenitors cultured at ambient oxygen tensions may lead to decreased progenitor survival through increased oxidative degradation of cell membranes and intracellular hemoglobin, processes normally controlled in erythrocytes by glutathione peroxidase. The adverse effect of ROS on erythroid progenitors is further supported by the observation that the addition of antioxidants to the media enhances erythroid progenitor survival at ambient oxygen tensions. For this reason, the methyl cellulose formulation used in CFAs of the present invention includes 2-mercaptoethanol as an antioxidant. Other antioxidants, including reduced glutathione, mannitol and α-tocopherol have also been reported to enhance erythroid progenitor survival (Ono and Alter, Exp. Hematol. 23:1372-77, 1995; Meagher et al., Blood 72:273-81, 1988; Rich and Kubanek, Br. J. Hematol. 52:579-88, 1982).

The present invention demonstrates that, consistent with previous reports, more erythroid progenitor colonies form at the physiological 5% O_2 than at ambient oxygen tensions. Presumably the production of ROS is reduced at 5% O_2 thus accounting for the

increased erythroid progenitor survival. Furthermore, the present invention shows that HAo, HEMOLINK™ and hemin, but not $FeCl_3$, can stimulate an even greater increase in the number of erythroid progenitor colonies at lower oxygen tensions. One potential mechanism for the enhanced survival of erythroid progenitors in the presence of HAo, HEMOLINK™ and hemin at low oxygen tensions may be through the ability of these agents to act as antioxidants themselves. Indeed, the exogenously added hemoglobin used in the present invention may reduce the level of ROS through its own oxidation and may also provide an oxygen buffering capacity to developing erythroid cells. It has previously been reported that the growth promoting effects of hemin and low oxygen tensions on erythroid progenitors are additive, indicating an independent mechanisms of action for these two agents (Weinberg et al., Hemoglobin 19:263-75, 1995). Thus, it is unlikely that the increase in erythroid progenitors observed in the presence of hemin is due solely to its property as an antioxidant. The ability of hemoglobin to stimulate a similar increase in erythroid progenitor colonies as seen in response to hemin also indicates that hemoglobin does not elicit this response by merely functioning as an antioxidant. The lack of promotion of erythroid progenitor growth by $FeCl_3$ at low oxygen tensions may be attributed to the well known activity of iron in catalyzing the formation of ROS. These data indicate that HAo and HEMOLINK™ are effective in stimulating erythropoiesis under physiological conditions.

Hemoglobin Stimulates Erythroid Progenitors at Low Concentrations of Erythropoietin

A particularly preferred embodiment of the present invention is the stimulation of erythroid progenitors by hemoglobin in the presence of reduced concentrations of erythropoietin. The Epo used in the experimental work reported herein is recombinant human Epo (tissue culture grade) obtained from R&D Systems (catalog number 286-EP), although any biologically active form of Epo should work equally well. Quantities are expressed in international activity units (U) per ml of solution. It is used in the form of a sterile-filtered solution in 50% PBS with carrier. Normally 2 U/ml Epo is used to stimulate maximal progenitor growth in the CFA. To determine the erythropoietic activity of

hemoglobin under submaximal Epo stimulation, the effects of HAo (15.6 μM), HEMOLINK™ (15.6 μM), hemin (100 μM) and $FeCl_3$ (250 μM) were tested in the presence of 0.2 and 0 U/ml Epo at both ambient and 5% O_2. Few colonies form on the control plates (vehicle only) containing only 0.2 U/ml Epo. The pale pink color of these colonies indicates that they are poorly hemoglobinized. Surprisingly, hemin, HAo and HEMOLINK™, but not $FeCl_3$, all increase the number of BFU-E colonies in the presence of only 0.2 U/ml Epo at ambient oxygen tensions. Similar results are observed at 5% O_2 under which conditions more erythroid progenitor colonies are produced than at ambient oxygen tensions. The dark red color of colonies which form in the presence of HAo, HEMOLINK™ or hemin at 0.2 U/ml Epo indicates that they are much more highly hemoglobinized than the control colonies. These data show that hemoglobin can promote Epo- induced erythroid progenitor proliferation (increased colony number) and differentiation (increased hemoglobinization of the colonies). No erythroid colonies were observed under any conditions in the absence of Epo. The reported role of Epo in inducing only differentiation and prolonging the G1 or quiescent phase of the erythroid cell cycle at low doses suggests that hemoglobin is capable of serving as a mitogen and providing a necessary and complementary proliferative signal that is otherwise only provided by high doses of Epo. Thus, Epo is essential for erythroid progenitor growth but hemoglobin can considerably lower the amount of Epo required to achieve an effective erythropoietic response. This discovery has important therapeutic applications for the use of hemoglobin in the treatment of anemias due to decreased Epo production, either alone or in conjunction with conventional Epo therapy.

The present invention demonstrates that the stimulation of erythroid progenitors derived from umbilical cord blood which is enriched in hematopoietic progenitors. It also demonstrates that hematopoietic progenitors derived from adult peripheral blood equally are stimulated by hemoglobin, and that the stimulation of adult progenitors by hemoglobin is also specific for erythroid progenitors, increased at low oxygen

tensions and synergizes with low concentrations of erythropoietin. Thus, hemoglobin stimulates erythroid progenitors from any hematopoietic tissue including blood and bone marrow.

The invention further demonstrates that primitive stem cells characterized by the presence of the hematopoietic progenitor marker CD34 and lacking either CD38 or CD33 differentiation antigens are also stimulated by hemoglobin. Such highly purified stem cells are multipotential and substantially free of contaminating committed progenitor and nonprogenitor cells. Thus, hemoglobin directly stimulates primitive hematopoietic progenitors and, in concert with other cytokines such as IL-3 and Epo, promotes the preferential proliferation and differentiation of cells of the erythroid lineage. The results obtained using highly purified progenitor cells indicates that no accessory cell population is involved and that the effects of hemoglobin are direct and not mediated indirectly through the stimulation of accessory cells that in turn stimulate erythroid progenitors via direct contact or a soluble factors(s).

Hemoglobin in the Treatment of Anemia

A preferred embodiment of the present invention is the therapeutic application of hemoglobin to the treatment of anemia. HEMOLINK™ is a hemoglobin based oxygen carrier intended for use as a red blood cell substitute at doses that may exceed 100 g. It has been demonstrated to be safe in a series of animal experiments as well as in a Phase I clinical trial in humans. The present invention specifically proposes the repetitive administration of HEMOLINK™ in small doses to patients with anemia of any type. The anemias most amenable to hemoglobin therapy include: (1) anemias due to insufficient Epo production, including chronic renal failure, malaria, AIDS, rheumatoid arthritis, anemia of cancer, sickle cell anemia, prematurity and late anemia associated with Rhesus hemolytic disease of the newborn; (2) anemias due to inadequate iron incorporation, including iron-deficient anemias and anemias associated with chronic disease (*e.g.* infection, inflammation, trauma, or

neoplastic diseases); and (3) anemias due to bone marrow failure that may be idiopathic or drug-induced.

This invention demonstrates that hemoglobin can partially substitute for Epo in the stimulation of erythropoiesis. Using the colony formation assay, comparable levels of erythroid progenitors proliferated in one-tenth the amount of Epo typically used (0.2 U/ml vs. 2 U/ml) when 1.0 mg/ml of purified hemoglobin, native or crosslinked, was added to the culture. Thus, any anemia which can be treated by Epo therapy could theoretically be managed by the combination therapy of reduced Epo doses supplemented with hemoglobin administration. Indications which are currently being treated with exogenous Epo, and thus those which are amenable to Epo/hemoglobin combination therapy, include end stage renal disease (including chronic renal failure), anemias associated with rheumatoid arthritis, cisplatin-associated anemia, solid tumors, lymphomas treated with or without chemotherapy, multiple myeloma, AIDS or myelodysplastic disorders. Epo therapy has also been used in patients undergoing allogeneic bone marrow transplants (Sowade, Blood 89:411-18, 1997) and infants suffering from late anemia associated with Rhesus hemolytic disease (Zachee, Drugs 49:536-47, 1995). Recently, Epo therapy has been granted U. S. approval for use in anemic patients scheduled to undergo elective, non-cardiac, non-vascular surgery to reduce the need for allogeneic blood transfusions (SCRIP, 2194:19, 1997). Other conditions which are associated with low endogenous Epo levels but are not currently being treated with exogenous Epo include malaria-induced anemia (Burgmann, Am. J. Trop. Med. Hyg. 54:280-83, 1996) and premature neonates (Zachee, Drugs 49:536-47, 1995). These conditions can also benefit from combination Epo/hemoglobin therapy.

Hemoglobin Reduces the Amount of Epo Required to Treat of Anemia

The current regiment for the treatment of anemia with exogenous Epo is to achieve a hematocrit between 30 - 36% (Dunn and Markham, Drugs 51:299-318, 1996). Presently, the safety and added benefits of maintaining the hematocrit between 39 - 45% (corresponding to normal levels) is being assessed for Epo therapy. The amount of Epo

administered for the treatment of anemia is dependent on the type of anemia, the route of Epo administration and the level of endogenous Epo. Epo doses of 225 U/kg/week administered three times weekly or 429 U/kg/week administered once a week have been reported to maintain a hematocrit of 33 - 40% in patients with end-stage renal disease. Assuming an 85 ml blood volume/kg these doses range from 0.9 - 5.1 U/ml in the blood volume. Based on the demonstration that *in vitro* hemoglobin (1.0 mg/ml) can compensate for a reduction in Epo dose from 2 to 0.2 U/ml, then Epo administration could be reduced to 0.27 - 0.51 U/ml (equivalent to 22.5 or 42.9 U/kg/week) if supplemented with 1.0 mg/ml hemoglobin (equivalent to 85 mg/kg/week). Examples of other disorders currently being treated with exogenous Epo and typical Epo doses which are administered to these patients are listed in Table 2.

Table 2
The Dosages of Erythropoietin used to Treat Anemia

Disorder	Epo Dose Administered
rheumatoid arthritis* cisplatin-associated anemia* solid tumors* lymphomas (± chemotherapy)* multiple myeloma*	150 U/kg (1.8 U/ml) s.c. daily or 3x weekly
AIDS*	starting dose: 100 - 200 U/kg (1.2 - 2.4 U/ml) 3x weekly
allogeneic bone marrow transplant*	1400 U/kg/week (16.5 U/ml/week)
myelodysplastic disorder*	150 - 1800 U/kg/week (1.8 - 21.2 U/ml/week)
Epo therapy to reduce subsequent allogeneic blood transfusions in elective surgery	300 U/kg/day (3.5 U/ml/kg) s.c. for 10 days before surgery, on the day of surgery, and 4 days post-surgery 600 U/kg (7 U/ml) s.c. in weekly doses, 3, 2, and 1 week before surgery and on the day of surgery
late anemia in Rhesus hemolytic disease**	200 U/kg (2.4 U/ml) 3x per week for 6 weeks

* Zachee, Drugs 49:536-47, 1995
** Ovali et al., Pediatr. Res. 39:831-34, 1996

The doses of Epo used for the treatment of the disorders in Table 2 are comparable to those used for the treatment of chronic renal failure and thus these respective anemias should be amenable to Epo/hemoglobin combination therapy.

Another strategy for the co-administration of Epo and hemoglobin is to reduce the number of Epo administrations required during therapy. Several Epo therapies follow the regimen of three weekly injections, either intravenously or subcutaneously. Multiple low dose or single high dose injections of Epo are required due to its relatively short half-life *in vivo*. The half-life of Epo in plasma has been estimated to be 5.6 - 8.8 hours after an intravenous injection and 11.2 - 21.1 hours after a subcutaneous injection, however only 21.5 - 46.6% of the Epo administered subcutaneously is actually absorbed into the bloodstream (Dunn and Markham, Drugs 51:299-318, 1996). HEMOLINK™ has a longer half-life. At therapeutic doses of 0.4 g HEMOLINK™/kg body weight the half-life of the 64 kD fraction, which represents ~33% of the crosslinked hemoglobin species, is ~7 hours whereas that of the >64 kD fraction, representing ~66% of the crosslinked hemoglobin species, is ~21 hours (Table 3). Longer half-life of the cross-linked hemoglobin can therefore compensate for Epo which may decline at a much faster rate. Thus, hemoglobin co-administered with Epo could prolong the effectiveness of Epo and therefore this combination therapy can be used to reduce the required frequency of Epo administrations.

TABLE 3

Half-Life of Epo versus HEMOLINK™

Hours post administration	*Epo, U/ml	*HEMOLINK™, mg/ml
0	2.7	4.7
21	0.33	1.75
42	0.041	0.81
63	0.0052	0.39
84	0.00065	0.19

Epo: single i.v. administration of 225 U/kg Epo (2.7 U/ml assuming 85 ml/kg)
HEMOLINK™: single i.v. administration of 4 ml/kg HEMOLINK™ (4.7 mg/ml)
HEMOLINK™: 33% 64 kD: half-life = 7 hours; 66% > 64 kD: half-life = 21 hours

Hemoglobin in the Treatment of Iron-Deficient Anemia

Since hemoglobin is an effective source of heme iron, hemoglobin has potential in the treatment of iron-deficient anemias, and thereby provides a unique dual function in the stimulation of erythropoiesis: iron delivery and erythroid progenitor stimulation. The effectiveness of hemoglobin both in providing iron and in stimulating erythropoiesis has been demonstrated *in vivo* in the present invention in iron-deficient anemic rats. HEMOLINK™ is more effective than parental iron in treating iron-deficient anemia in animals where it efficiently stimulates erythropoiesis without increasing serum iron levels. HEMOLINK™ could be used to treat anemias that result from inadequate heme synthesis including iron-deficient anemias, sideroblastic anemias which result from intracellular iron accumulation and anemias associated with chronic disease which result from abnormal sequestration of iron. The present invention shows that hemoglobin directly stimulates erythroid progenitors and efficiently delivers heme that both stimulates the cells and is available for incorporation into newly synthesized hemoglobin. In addition, the anemias of chronic disease are often associated with an inadequate increase in Epo in response to the degree of anemia. Hemoglobin could be especially effective in the treatment of such anemias via its dual ability to synergize with Epo and provide heme.

Hemoglobin in the Treatment of Bone Marrow Failure or Suppression

The present invention demonstrates that hemoglobin directly stimulates primitive multipotential stem cells and in concert with IL-3 and Epo promotes the preferential proliferation and differentiation of primitive progenitors of the erythroid lineage. Anemias that result from bone marrow failure such as idiopathic or drug-induced aplastic anemia, bone marrow suppression or ablation via chemo- and/or radiotherapy, or from delayed engraftment following bone marrow or stem cell transplantation may be treated by the administration of a suitable amount of hemoglobin. Such treatments would be used alone or in concert with other conventional treatments including cytokines such as G-CSF, GM-

CSF, thrombopoietin and Epo to specifically increase the rate of erythroid progenitor recovery, and the reconstitution of red blood cells and bone marrow.

Hemoglobin in the Delivery of Heme

A significant portion of the activity of hemoglobin in the stimulation of erythropoiesis can be attributed to the delivery of heme. The present invention shows that hemoglobin is as effective as hemin in the stimulation of erythroid progenitors and is a better inducer of erythroid differentiation than hemin. Not only does hemin stimulate erythroid progenitor proliferation and globin synthesis, it (as heme) is an essential component in the synthesis of hemoglobin. Heme is required by several other cell types for the generation of various heme-containing proteins, including myoglobin, the cytochromes and a variety of enzymes. Thus, any requirement for, or defect in the synthesis of, heme that is treatable by exogenous heme administration can be effectively treated by the administration of hemoglobin. By way of example, acute hepatic porphyria result from inherited abnormalities in specific enzymes of the heme synthetic pathway (analogous to the succinyl acetone inhibition described in the present invention) leading to the accumulation of heme precursors (Bissell, J. Hepatol. 6:1-7, 1988). The goal of therapy for these diseases is the replenishment of cellular heme. Because of the poor solubility of heme at neutral pH, heme derivatives such as hematin (hydroxyheme) have been used in patients and shown to be effective in the treatment of acute porphyria (Bissell, J. Hepatol. 6:1-7, 1988). Hematin is unstable and possesses numerous toxic side-effects (Goetsch and Bissell, New. Engl. J. Med. 315:235-38, 1986; Cannon et al., PDA J. Pharm. Sci. Technol. 49:77-82, 1995). However, hemoglobin is a natural and physiological carrier and stabilizer of heme. Moreover, the endogenous haptoglobin-hemoglobin system of humans efficiently and specifically delivers hemoglobin to liver cells. The lower overall heme requirement of liver cells than hemoglobin synthesizing cells would permit lower doses of hemoglobin to be used to treat the heme deficiency in these cells. Thus, the same erythropoietic promoting effects of hemoglobin which are in part mediated by the delivery of heme can be utilized to treat other disorders

in which heme delivery is important. The relative low toxicity of crosslinked hemoglobin and its ability to increase the solubility and stability of heme make it an ideal heme delivery vehicle.

The following examples illustrate embodiments of the invention, but should not be viewed as limiting the scope of the invention.

Examples

Example 1 Purified Adult Hemoglobin, HEMOLINK™, Hemin and $FeCl_3$ Support the Expansion of Erythroid Progenitors from Umbilical Cord Blood

Adult hemoglobin was purified according to patents 5,439,591 and 5,545,328 to obtain HAo and HEMOLINK™ was prepared from HAo according to U.S. patent 5,532,352. Human umbilical cord blood intended for discard was collected from the umbilical vein of placentas immediately postpartum into 10 ml vacutainer tubes containing 143 USP units/ml sodium heparin. Low density mononuclear cells were separated from red blood cells by centrifugation over a 1.077 g/ml density gradient. Cells removed from the plasma/density gradient interface were incubated overnight in a tissue culture flask with 10 ml cell culture medium containing 10% fetal bovine serum, and non-adherent LDMNC were further purified with an additional density gradient step. The LDMNC were then plated into colony formation assays (CFA) on the second day of isolation. In the CFA, LDMNC were plated at a density of 1×10^5 cells/ml in Iscoves modified Dulbeccos cell culture medium containing 0.8% methyl-cellulose, 30% fetal bovine serum, 1% bovine serum albumin, 0.1 mM 2-mercaptoethanol and 2 mM L-glutamine. Unless indicated otherwise, under standard CFA conditions 2 U/ml Epo and 10 ng/ml IL-3 are added to the formulation to stimulate hematopoietic progenitor growth, specifically BFU-E, CFU-E and CFU-GM.

HAo, HEMOLINK™, hemin and $FeCl_3$ were added to the cultures at the concentrations indicated in Table 4. Each condition was tested in duplicate in from 2 - 18 independent experiments. Cultures were maintained in a humidified incubator at 37°C, 5%

CO_2 and ambient oxygen tensions. The number of hematopoietic progenitors was scored between days 13 - 15 by counting the number and types of colonies present.

The mean fold increase in cord blood BFU-E, CFU-E, and CFU-GM progenitor number produced by 1.0 mg/ml HAo (15.6 μM; n = 18) or HEMOLINK™ (15.6 μM; n = 6), 100 μM hemin (n = 17 - 18) or 250 μM $FeCl_3$ (n = 8) is shown in Figure 1. As indicated, 1.0 mg/ml HAo stimulated a 3.2-fold ($p < 0.01$) increase in BFU-E and a 1.8-fold increase in CFU-E ($p < 0.01$). The fold increase in colonies is expressed relative to control cells treated only with the respective diluents (vehicles) used in the preparation of the test compounds (fold expansion = 1.0; *$p < 0.05$, ** $p < 0.01$). The fold increase in progenitor number was dose-dependent over the range 10 - 1000 μg/ml HAo (Table 4) reaching maximum erythroid progenitor stimulation at 1.0 mg/ml HAo (15.6 μM). HEMOLINK™, at 1.0 mg/ml (15.6 μM) produced a similar increase in progenitor number to 1.0 mg/ml HAo. Hemin and $FeCl_3$ also showed a dose-dependent increase in the stimulation of erythroid progenitors, reaching their respective maxima at 100 μM. Although HAo, HEMOLINK™ and hemin, but not $FeCl_3$, stimulated a significant increase in CFU-E, the overall magnitude of CFU-E stimulation was much less than obtained for BFU-E. CFU-GM numbers were unaffected by $FeCl_3$ and hemin. The apparent decrease in CFU-GM treated with HAo and HEMOLINK™ results from an under-estimation of CFU-GM numbers due to the formation of white granular hemoglobin precipitates in the CFA which render nonhemoglobinized colonies difficult to score. As indicated in Table 4, CFU-GM numbers were only decreased at the highest HAo or HEMOLINK™ concentrations where hemoglobin precipitation was greatest.

TABLE 4

Effect of $FeCl_3$, Hemin, HAo and HEMOLINK™ on Progenitor Number

		Progenitor Type					
Agent	Dose	BFU-E	n	CFU-E	n	CFU-GM	n
$FeCl_3$, μM	1.0	1.2	2	1.0	2	1.2	2
	10	1.4	2	1.2	2	1.0	2
	100	* 2.1	5	1.2	5	0.8	5
	250	* 1.7	8	1.1	8	1.3	8
Hemin, μM	1.0	0.9	5	1.2	5	1.5	4
	10	1.5	5	* 1.6	5	1.4	4
	64	* 2.3	6	1.8	6	1.2	5
	100	** 2.9	18	** 2.0	18	1.0	17
	250	2.2	4	2.2	4	1.4	4
HAo, μg/ml	10	1.1	5	1.2	5	1.4	4
	100	1.3	5	1.5	5	1.5	4
	500	** 1.7	11	* 1.4	11	1.2	9
	1000	** 3.2	18	** 1.8	18	** 0.5	18
HEMOLINK™, μg/ml	1000	* 2.8	6	* 1.4	6	* 0.6	6

* $p < 0.05$, ** $p < 0.01$

Example 2 Enhanced Growth of Cord Blood Erythroid Progenitors Stimulated by HAo, HEMOLINK™ and Hemin is further Enhanced at Low Oxygen Tension

Erythroid progenitor colony formation assays were conducted as described in Example 1 except that cultures were incubated at either ambient oxygen tensions (~20% O_2) or 5% O_2. The reduced oxygen tension of 5% O_2 approximates the physiological intravascular oxygen tension in mammals. The mean fold increase in cord blood BFU-E, CFU-E and CFU-GM progenitor number at 5% O_2 produced by 1.0 mg/ml HAo (15.6 μM; n = 11) or HEMOLINK™ (15.6 μM; n = 6), 100 μM hemin (n = 10), 250 μM $FeCl_3$ (n = 6) or no addition (n = 17 - 18), is shown Figure 2. Results are expressed relative to control cells treated only with the respective vehicles and cultured at ambient oxygen tensions (~20% O_2; fold expansion = 1.0; *$p < 0.05$, ** $p < 0.01$).

More erythroid progenitor colonies formed in the untreated controls at 5% O_2 than at ambient oxygen tensions (3.8-fold and 2-fold increase in BFU-E and CFU-E, respectively). The oxygen-dependent increase in erythroid progenitors was further enhanced in the presence of HAo, HEMOLINK™ or hemin. Again these three agents stimulated comparable increases in BFU-E and CFU-E (~8-fold and ~3-fold, respectively) relative to the untreated controls at ambient oxygen tensions. $FeCl_3$ did not increase the fold expansion of erythroid progenitors beyond that of the untreated controls at 5% O_2 (no addition).

Example 3 HAo and HEMOLINK™ Promote Cord Blood Erythroid Progenitor Proliferation under Reduced EPO Concentrations

Erythroid progenitor colony formation assays were conducted as described in Example 1 except that cultures were initiated in the presence of Epo concentrations ranging from 0 - 2 U/ml. The mean fold increase in cord blood BFU-E, CFU-E and CFU-GM progenitor number produced by 1.0 mg/ml HAo (15.6 μM; n = 9) or HEMOLINK™ (15.6 μM; n = 6), 100 μM hemin (n = 8), 250 μM $FeCl_3$ (n = 6), or no addition (n = 9 - 10) in the presence of reduced Epo concentrations (0.2 U/ml) is shown in Figure 3. Cultures were maintained at ambient oxygen tensions (~20% O_2) for 14 days. Results are expressed relative to control cells at 2 U/ml Epo treated only with the respective vehicles (solid line at fold expansion = 1.0; *$p < 0.05$, ** $p < 0$ 01). Erythroid progenitor colonies were undetectable in the absence of Epo.

Reducing the Epo concentration 10-fold from the standard concentration of 2 U/ml to 0.2 U/ml resulted in a 5-fold decrease in BFU-E progenitors. The addition of hemin to such plates, generated the same number of BFU-E as the untreated controls at 2 U/ml Epo, and HAo and HEMOLINK™, but not $FeCl_3$, stimulated a 3-fold increase in BFU-E number over the untreated controls (no addition) at 0.2 U/ml Epo. Hemin, HAo or HEMOLINK™ could not support the growth of erythroid progenitor colonies in the absence of Epo, indicating the specificity of these agents for the erythroid lineage.

Example 4 HAo and HEMOLINK™ Promote Cord Blood Erythroid Progenitor Proliferation in the Presence of Reduced EPO Concentrations at Low Oxygen

Erythroid progenitor colony formation assays were conducted as described in Example 3 except that cultures were incubated at 5% O_2. The mean fold increase in cord blood BFU-E, CFU-E and CFU-GM progenitor number at 5% O_2 produced by 1.0 mg/ml HAo (15.6 μM; n = 9) or HEMOLINK™ (15.6 μM; n=6), 100 μM hemin (n = 8) or 250 μM $FeCl_3$ (n=6) or no addition (n = 9 - 10) in the presence of reduced Epo concentrations (0.2 U/ml) is shown in Figure 4. Cultures were maintained at 5% O_2 for 14 days. Results are expressed relative to control cells at 2 U/ml Epo treated only with the respective vehicles and cultured at ambient oxygen tensions (~20% O_2) for 14 days (solid line at fold expansion = 1.0; *$p < 0.05$). At 5% O_2, the number of erythroid progenitors was maintained in CFAs containing a 10-fold lower concentration of Epo (0.2 U/ml) than the standard dose of 2 U/ml. Under these conditions, 100 μM hemin stimulated a 4-fold and 3.2-fold increase in BFU-E and CFU-E colonies, respectively, as compared to the control plates at 2 U/ml Epo at ambient oxygen tensions. Similarly, 1.0 mg/ml HAo (15.6 μM) and HEMOLINK™ (15.6 μM) resulted in 3.3 and 2.6-fold increases in BFU-E, and 2.1 and 1.7-fold increases in CFU-E, respectively, over the controls. However, 250 μM $FeCl_3$ did not significantly increase the number of erythroid colonies stimulated under these conditions, similar to the results obtained with $FeCl_3$ at ambient oxygen tensions (Figure 3).

Example 5 Effect of Hemoglobin on the Proliferation of Erythroid Progenitors is not only Mediated through the Delivery of Heme

The effect of the various additives on the inhibitory effect of succinylacetone (SA), a heme synthesis inhibitor, on erythroid progenitors was determined. Primary LDMNC were cultured in methyl cellulose as described in Example 1. Cord blood LDMN were plated in the presence of 1.0 mg/ml HAo (15.6 μM; n = 12) or HEMOLINK™ (15.6 μM; n = 5), 100 μM hemin (n = 12), 250 μM $FeCl_3$ (n = 11) or no addition (n = 12), in the

absence or presence of 0.5 mM SA. Cultures were maintained in a humidified chamber at 37°C, 5% CO_2 and ambient oxygen tensions for 14 days.

Results are expressed as the number of total erythroid progenitors (BFU-E + CFU-E) present on plates treated with the various additives in the presence of SA relative to the number present on untreated plates (no SA or other addition). Significant differences in the presence of the respective additives compared to no addition (SA only; relative progenitor number = 0.31) are as indicated: *$p < 0.001$, **$p<0.0001$. Significant differences in the presence of the respective additives compared to no treatment (no SA; solid line at relative erythroid progenitor number = 1.0) are as indicated: + $p < 0.05$, ++ $p < 0.001$.

As shown in Figure 5, SA alone had an inhibitory effect on the growth of erythroid progenitors (BFU-E + CFU-E). 100 μM hemin was able to overcome the inhibitory effect of SA on erythroid progenitors. In the presence of hemin, SA had a slight inhibitory effect on the number of BFU-E, but no effect on the number of CFU-E. Although 15.6 μM HAo (64 μM heme equivalents) could partially reverse the SA toxicity, unlike 100 μM hemin, both HAo and HEMOLINK™ were unable to fully restore erythroid progenitor numbers. These data support a role for hemoglobin in the stimulation of erythroid progenitors that is more than just the delivery of heme. $FeCl_3$, was also unable to overcome the inhibitory effect of SA. Succinylacetone did not inhibit CFU-GM proliferation illustrating the specificity of this inhibitor for erythroid progenitors.

Example 6 Erythroid Colonies which form in the Presence of HAo and HEMOLINK™ are Larger and Produce more Hemoglobin per Cell

Shown in Figure 6 are photographs of representative cord blood BFU-E colonies which form in CFA at day 14 in the presence of 2 U/ml Epo at 5% O_2 under the following conditions: (a) no addition (vehicle only), (b) 100 μM hemin and (c) 1.0 mg/ml (15.6 μM) HAo. Photographs also include representative colonies that formed in the presence of 0.2 U/ml Epo and 5% O_2, under the following conditions: (d) no addition

(vehicle only), (e) 100 μM hemin and (f) 1.0 mg/ml (15.6 μM) HEMOLINK™. Erythroid colonies which formed in the presence of HAo or HEMOLINK™ were larger and redder than colonies on the untreated control plates (vehicle only) or those to which hemin was added. These observations are consistent at both 2 and 0.2 U/ml Epo and at either ambient or 5% O_2. The colonies which formed in the presence of hemin (100 μM) under similar Epo concentrations and oxygen tensions were mostly smaller and less red in color than HAo- or HEMOLINK™-treated cells. The pale pink erythroid colonies present at 0.2 U/ml Epo in the control plates indicates poor hemoglobin synthesis whereas the dark red colonies that formed in the presence of HAo or HEMOLINK™ indicates the stimulation of significantly greater hemoglobin synthesis by exogenous hemoglobin.

Hemoglobin synthesis in erythroid colonies was analyzed by anion exchange high performance liquid chromatography (HPLC). Colonies were harvested from CFA plates and cell lysates were prepared after the progenitor colonies had been enumerated. Colonies were isolated from methyl cellulose by several washes in PBS. The cells were pelleted and lysed in 50 mM Tris, pH 8.8 and the cell debris removed by pelleting the cell lysate. The lysate supernatants were filtered through a 0.2 μm filter prior to loading onto a POROS® HQ/H anion exchange HPLC column (PerSeptive Biosystems). The hemoglobin was eluted with an increasing NaCl gradient and the optical density (O.D.) monitored at a wavelength of 414 nm. The amount of hemoglobin present in the lysates was quantitated by comparison with standard hemoglobin solutions of adult (HbA) and fetal hemoglobin (HbF).

Representative HPLC profiles for hemoglobin produced by erythroid colonies that formed at 5% O_2 in the presence of 2 U/ml Epo with no addition (vehicle only), 100 μM hemin, 1.0 mg/ml HAo (15.6 μM) HAo, 1.0 mg/ml HEMOLINK™ (15.6 μM), or 250 μM $FeCl_3$ are shown in Figure 7. The amount of hemoglobin produced under the above conditions is shown in Table 5.

TABLE 5

Effect of Hemin, HAo, HEMOLINK™ and $FeCl_3$ on Hemoglobin Production

Condition	adult Hb, μg	fetal Hb, μg	total Hb, μg
no addition	3.7	4.4	8.1
100 μM hemin	8.7	19.4	28.1
1.0 mg/ml HAo	22.6	33.1	55.7
1.0 mg/ml HEMOLINK™	20.5	28.0	48.5
250 μM $FeCl_3$	5.0	9.5	14.5

Erythroid progenitors which formed in the presence of HAo or HEMOLINK™ contained the most hemoglobin. Both adult and fetal hemoglobin synthesis increased in response to HAo or HEMOLINK™. To exclude the possibility that the increase in adult hemoglobin was not simply due to contamination of the cell lysates with the exogenously added hemoglobin, HPLC analysis was conducted on cell lysates prepared from CFAs which contained 0 U/ml Epo, 1.0 mg/ml HAo or HEMOLINK™, and maintained at 5% O_2. Erythroid colonies did not form under these conditions. Hemoglobin was undetectable in these cell lysates suggesting that the added HAo or HEMOLINK™ is adequately washed away, or removed from the cells and does not contaminate the cell lysates.

The amount of hemoglobin produced per erythroid cell was estimated by assuming 30,000 cells/BFU-E and 100 cells/CFU-E. Table 6 shows the amount of hemoglobin (pg) produced per cell from the above cultures.

TABLE 6

Effect of Hemin, HAo, HEMOLINK™ and $FeCl_3$ on Hemoglobin Production per Cell

Condition	adult Hb, pg/cell	fetal Hb, pg/cell	total Hb, pg/cell
no addition	0.16	0.19	0.35
100 μM hemin	0.14	0.32	0.46
1.0 mg/ml HAo	0.37	0.54	0.91
1.0 mg/ml HEMOLINK™	0.38	0.51	0.89
250 μM $FeCl_3$	0.14	0.26	0.40

Thus, the increased redness of the erythroid colonies that formed in the presence of HAo and HEMOLINK™, as compared to those in the presence of hemin or $FeCl_3$, corresponds to an increase in hemoglobin production. These data suggest that exogenously added hemoglobin can directly stimulate the endogenous synthesis of both adult and fetal hemoglobin by erythroid cells.

Example 7 HAo Enhances the Differentiation of Erythroid Cells in Liquid Culture

The effect of HAo on erythroid differentiation was tested on cord blood LDMNC. LDMNC were isolated as described in Example 1 and maintained in a liquid culture system that supports the expansion and differentiation of erythroid cells. 1.0 mg/ml HAo (15.6 μM) was added to Epo-stimulated cultures and the cultured cells were analyzed by flow cytometry (Epics Elite, Coulter) after 20 days for the co-expression of the red cell-specific marker, glycophorin A, and the transferrin receptor, CD71. Co-expression of these two antigens is indicative of erythroid cell differentiation. Cultured cells were stained with anti-CD71 (abscissa) and anti-glycophorin A (ordinate) antibodies and the number of single- or double-stained cells (z-axis) analyzed. Cells simultaneously expressing both red blood cell membrane markers are located in the upper right region of each plot while those cells expressing neither antigen are located in the lower left region. The percentage of glycophorin $A^+/CD71^+$ cells was determined from analysis of at least 6,500 cells per sample. As demonstrated in Figure 8, HAo increased the percentage of cells co-expressing glycophorin A and CD71 compared to untreated cultures (80% vs. 53%, respectively), indicating that hemoglobin stimulates erythroid cell differentiation in addition to erythroid progenitor proliferation. These data also demonstrate that hemoglobin stimulates erythroid-specific gene expression and that exogenous hemoglobin is not simply used as a raw material in the production of new hemoglobin by differentiating erythroid progenitors.

Example 8 Purified Adult Hemoglobin, HEMOLINK™, Hemin and $FeCl_3$ Support the Expansion of Erythroid Progenitors derived from Adult Peripheral Blood

Blood was collected with informed consent from the antecubital vein of healthy adult human volunteers into vacutainers containing 143 USP U/ml sodium heparin. Low density mononuclear cells (LDMNC) were isolated by centrifugation over a density gradient as described in Example 1. Plastic adherent cells were removed by an overnight incubation in a tissue culture flask and the non-adherent cells were placed directly in a CFA. Conditions for the CFA are similar to those described in Examples 1 - 4.

The mean fold increase in adult blood BFU-E, CFU-E and CFU-GM progenitor number produced by 1.0 mg/ml HAo (15.6 μM) or HEMOLINK™ (15.6 μM), 100 μM hemin or 250 μM $FeCl_3$ is shown in Figure 9 (n = 3). Cultures were maintained at ambient oxygen tensions (~20% O_2) for 14 days. The fold increase in colonies is expressed relative to control cells treated only with the respective vehicles. All agents evaluated stimulated an increase in BFU-E number at ambient oxygen tensions. The magnitude of this increase was greater for adult than cord blood LDMNC. HAo, HEMOLINK™ and hemin stimulated comparable increases (~5 - 6-fold) in BFU-E from adult blood LDMNC. $FeCl_3$ was less effective than HAo, HEMOLINK™ or hemin, and stimulated a <3-fold increase in BFU-E number. HAo, HEMOLINK™ and hemin, but not $FeCl_3$, also stimulated slight increases in the number of CFU-E progenitors.

The mean fold increase in adult blood BFU-E, CFU-E and CFU-GM progenitor number at 5% O_2 is shown in Figure 10 with the same additives. Results are expressed relative to control cells treated only with the respective vehicles and cultured at ambient oxygen tensions (~20% O_2). Reduced oxygen tensions (5% O_2) enhanced the growth of BFU-E which was further increased by the addition of HAo, HEMOLINK™, hemin or $FeCl_3$. All agents stimulated a similar increase in BFU-E number (~9 - 11-fold) at 5% O_2 compared to untreated control cells at ambient oxygen tensions.

Mean fold increase in adult blood BFU-E, CFU-E and CFU-GM progenitor number in the presence of reduced Epo (0.2 U/ml) (n=3) at ~20% O_2 is shown in Figure 11 with and without (no addition) the additives used in Figure 9. Results are expressed relative to control cells at 2 U/ml Epo treated only with the respective vehicles (solid line at fold expansion = 1.0). Reducing the Epo concentration from the standard concentration of 2 U/ml to 0.2 U/ml resulted in a reduction in BFU-E and CFU-E number at ~20% O_2. As with cord blood, CFU-GM numbers were unaffected by reductions in Epo. The addition of HAo or HEMOLINK™ to CFAs containing 0.2 U/ml Epo stimulated ~4.5- and ~3.5-fold increases in BFU-E and CFU-E, respectively, compared to untreated controls (no addition), restoring the progenitor number to that obtained at 2 U/ml Epo. Hemin stimulated the greatest increase in BFU-E and CFU-E (~9- and ~6-fold, respectively) at 0.2 U/ml Epo versus the untreated controls (no addition). $FeCl_3$ stimulated only a ~2-fold increase in erythroid progenitor number at 0.2 U/ml Epo. HAo, HEMOLINK™, hemin and $FeCl_3$ could not support the growth of erythroid progenitors in the absence of Epo, indicating that Epo stimulation is essential for erythroid progenitor growth and differentiation.

Mean fold increase in adult blood BFU-E, CFU-E and CFU-GM progenitor number at 5% O_2 in the presence of reduced Epo (0.2 U/ml) (n = 3) is shown in Figure 12 with and without the additives used in Figure 9. Results are expressed relative to control cells at 2 U/ml Epo treated only with the respective vehicles and cultured at ambient oxygen tensions (~20% O_2) for 14 days (solid line at fold expansion = 1.0). The number of erythroid progenitors stimulated at 5% O_2 and 0.2 U/ml Epo was similar to that obtained with 2.0 U/ml Epo maintained at ambient oxygen tensions (no addition). The addition of HAo, HEMOLINK™, hemin, and to a lesser extent $FeCl_3$, stimulated further increases in erythroid progenitor number, particularly BFU-E, over the untreated controls (no addition) cultured under the same conditions.

Example 9 Purified Adult Hemoglobin, HEMOLINK™, Hemin and $FeCl_3$ support Expansion of Erythroid Progenitors from $CD34^+/CD38^-$ and $CD34^+/CD33^-$ Stem cells

$CD34^+/CD38^-$ cells were isolated from cord blood LDMNC using the STEMSEP™ system (StemCell Technologies Inc., Vancouver, Canada). The $CD34^+/CD38^-$ cell purity was increased from <0.1% in unfractionated LDMNC to ~81% $CD34^+/CD38^-$ cells post fractionation as determined by flow cytometry. To obtain the $CD34^+/CD33^-$ cells, $CD34^+$ cells were first isolated from cord blood LDMNC using the CEPRATE™ LC system (CellPro Inc., Bothell, WA). The $CD34^+/CD33^-$ fraction was then enriched to ~94% purity by flow fluorescence activated cell sorting (FACS; Epics Elite, Coulter Electronics, Hialeah, FL). The colony formation assay was conducted as described in Examples 1 - 4, except that 1×10^3 $CD34^+$ cells/ml were plated versus 1×10^5 unfractionated cord blood LDMNC/ml normally plated. Mean fold increase in BFU-E and CFU-E progenitor number produced from $CD34^+$ $CD38^-$ enriched (a; n = 2) and $CD34^+CD33^-$ enriched (b; n = 1) cord blood LDMNC stimulated by 1.0 mg/ml HAo (15.6 μM) or HEMOLINK™ (15.6 μM), 100 μM hemin, 250 μM $FeCl_3$ in the presence of 2 U/ml Epo is shown in Figure 13. Cultures were maintained at 5% O_2 for 14 days. The results are expressed relative to control cells treated only with the respective vehicles and cultured under the same conditions.

For both enriched progenitor cell populations, HAo, HEMOLINK™, hemin and to a lesser extent $FeCl_3$, stimulated an increase in BFU-E, as compared to untreated control cells cultured under the same conditions. The CFU-E numbers were not affected by the addition of any of the agents.

Example 10 Treatment of Anemia by Hemoglobin Infusion

HEMOLINK™ was evaluated for the stimulation of erythropoiesis in anemic rats. Male Sprague Dawley rats were made anemic on an iron deficient diet over a 4 week period. Six of the anemic rats received two infusions via tail vein injection one week apart

(dose I and dose II) of 10% of total blood volume of 9.8 g/dL HEMOLINK™. Nine anemic rats received an equivalent amount of parenteral iron (INFUFER™, Sabex) per kg body weight for comparison. Both solutions contained 342.9 mg/L iron. The iron-deficient anemia of the HEMOLINK™ group was characterized by a pretreatment serum iron level of 10.0 mg/dL (n = 1), a reticulocyte count of 0.39 ± 0.08 x 10^{12}/L, a red blood cell count of 1.85 ± 0.35 x 10^{12}/L, a 6.5 ± 1.26 % hematocrit and a total blood hemoglobin of 30.5 ± 4.38 g/L.

Figure 14 shows that the mean increase in a) the number of reticulocytes, b) the number of red blood cells, c) the hematocrit and d) the total hemoglobin concentration in anemic male rats treated with HEMOLINK™ (n = 6) versus INFUFER™ (*i.e.* iron; n = 9). HEMOLINK™ induced a significant increase in reticulocytes, red blood cells, hematocrit and total hemoglobin over that induced by INFUFER™ (*p <0.05 compared with INFUFER™, ** p < 0.01 compared with pretreatment anemic rats). Reticulocyte count increases for male rats receiving HEMOLINK™ were observed 48 hours after each HEMOLINK™ infusion and reached statistical significance at 48, 216 and 336 hours post-infusion compared with the pretreatment value (p < 0.01). In contrast, the reticulocyte increase for male rats receiving equivalent amounts of parenteral iron were not statistically different from the pretreatment values at any time point. Comparison between the two male groups showed significantly greater increases in the reticulocyte counts at 216 and 336 hours after administration of HEMOLINK™ compared to INFUFER™. Anemic male rats infused with iron had only insignificant or no increases in red blood cell count, hematocrit or total hemoglobin.

Normal [225.0 ± 17.41 mg/dL (n = 4) for females vs. 131.25 ± 13.83 mg/dL (n = 4) for males] and anemic [22.5 mg/dL (n = 2) for females vs. 10.0 mg/dL (n = 1) for males] female rats appear to preserve higher levels of serum iron when compared with males treated and kept under the same conditions. At study termination, male rats which had received two infusions of HEMOLINK™ had a serum iron level of 10.0 ± 1.95 mg/dL (n

= 6). Anemic female rats receiving two doses of HEMOLINK™ had a serum iron level of 27.70 ± 9.88 mg/dL (n = 10). Anemic female rats showed a trend similar to the male rats, but gave an overall lower response to HEMOLINK™ which was not significantly different from INFUFER™. Thus, HEMOLINK™ is a stronger and more efficient stimulator of erythropoiesis in anemic rats than parenteral iron which does not lead to an overall increase in serum iron.

Example 11 Reduction in Toxicity of Ganciclovir

HEMOLINK™ was evaluated for its ability to protect erythroid progenitor cells from toxicity due to ganciclovir. Cord blood LDMNC were set up in CFAs in the presence and absence of 1 mg/ml HEMOLINK™ plus 0, 0.1, 1.0, 10, 50 or 100 μM ganciclovir. Conditions for the CFA were similar to those described in Examples 1-4. As shown in Figure 15, ganciclovir produces a dose-dependent inhibition of BFU-E, CFU-E and CFU-GM in the absence of HEMOLINK™. All data are expressed relative to the number of colonies of control plates without HEMOLINK™. The hematopoietic progenitors display different sensitivities to ganciclovir. CFU-E were unaffected by 1 μM ganciclovir. However, a similar inhibition of BFU-E occurs at a 50-fold lower dose of ganciclovir (1 μM). CFU-GM are similar to CFU-E and are more resistant to ganciclovir than to BFU-E, but unlike CFU-E, CFU-GM are completely inhibited at doses greater than 10 μM ganciclovir. HEMOLINK™ reduces toxicity of ganciclovir to BFU-E about 10-fold and also protects CFU-E. Not only were the colony numbers reduced in the presence of ganciclovir, colonies were very pale indicating poor hemoglobinization. In contrast, in the presence of HEMOLINK™, the more numerous erythroid colonies were much larger and redder. Due to the difficulty in scoring CFU-GM in the presence of hemoglobin precipitates, as previously noted, they could not be accurately measured under these conditions. Thus, HEMOLINK™ reduces toxicity of ganciclovir to erythroid progenitors with the most pronounced effect on BFU-E cells.

Example 12 Statistics

Statistical comparisons in Figures 1 - 4 and Table 4 are based on the Student's t-test, using meaningfully paired observations and a 2-tail rejection region. The general null hypothesis for the comparisons in Figures 1 - 4 is H_0 there is no significant difference in fold expansion of progenitors with no addition, HAo, HEMOLINK™, hemin or $FeCl_3$ at ambient or 5% O_2 and 2.0 or 0.2 Units of Epo versus the fold expansion of progenitors with no addition at ambient O_2 and 2.0 Units of Epo. Significant differences are indicated by * ($p < 0.05$) and ** ($p < 0.01$).

Statistical comparisons in Figure 5 are based on the Student's t-test, using meaningfully paired observations and a 1-tail rejection region. The general null hypotheses for the comparisons in Figure 5 are (1) "H_0^1": the inhibitory effect of SA on erythroid progenitor number is not reduced by HAo, HEMOLINK™, hemin or $FeCl_3$ when compared to the progenitor number measured under SA alone. Significant differences are indicated by * ($p < 0.001$) and ** ($p < 0.0001$); and (2) "H_0^2": the progenitor number produced by the effect of SA plus HAo, HEMOLINK™, hemin or $FeCl_3$ is at least as much as the progenitor number produced when there is no treatment (*i.e.* no addition and no SA). Significant differences are indicated by + ($p < 0.05$) and ++ ($p < 0.001$).

Statistical comparisons in Figure 14 are based on one way analyses of variance, followed by Bonefoni comparison post-hoc tests. The effect of HEMOLINK™ infusion on reticulocyte count, red blood cell count, total hemoglobin and hematocrit in anemic male rats was compared to iron infusion (INFUFER™; an iron-containing pharmaceutical composition) and to pretreatment levels. Significant differences are indicated by * $p < 0.05$ compared with INFUFER™ and ** $p < 0.01$ compared with pretreatment anemic rats.

Other embodiments and uses of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. All U.S. patents and patent applications, including provisional applications, and all other documents referenced herein, for whatever reason, are specifically incorporated by

reference. It is intended that the specification and examples be considered exemplary only, with the true scope and spirit of the invention being indicated by the following claims.

We Claim:

1. A method for the stimulation of erythropoiesis in the presence of reduced amounts of Epo comprising administering a composition comprising a heme-containing component to erythroid cells.

2. The method of claim 1 wherein the heme-containing component is selected from the group consisting of heme, hemin, hematin, hemoglobin, purified hemoglobin, recombinant hemoglobin, cross-linked hemoglobin, modified hemoglobin and mutated hemoglobin protein.

3. The method of claim 1 wherein the stimulation of erythropoiesis comprises the proliferation of erythroid stem cells.

4. The method of claim 1 wherein the erythroid stem cells are $CD34^+$ cells, $CD34^+/38^-$ cells or $CD34^+/33^-$ cells.

5. The method of claim 1 wherein the stimulation of erythropoiesis comprises proliferation of erythroid-progenitor cells.

6. The method of claim 3 wherein the erythroid-progenitor cells are CFU-GEMM cells, BFU-E cells or CFU-E cells.

7. The method of claim 6 wherein the stimulation of erythropoiesis occurs in the substantial absence of proliferation of myeloid-progenitor cells.

8. The method of claim 7 wherein the myeloid-progenitor cells are CFU-GM cells.

9. The method of claim 1 wherein the stimulation of erythropoiesis comprises the increased expression of hemoglobin from said erythroid cells.

10. The method of claim 9 wherein the increased expression of hemoglobin comprises the increased expression of adult and fetal hemoglobin.

11. The method of claim 1 wherein the composition is substantially free of hemoglobin protein.

12. The method of claim 11 wherein there is less than 1% hemoglobin protein in said composition.

13. The method of claim 1 further comprising administering Epo to said erythroid cells.

14. The method of claim 13 wherein the amount of Epo administered produces an effective concentration of less than about 5 U/ml.

15. A method for the stimulation of erythropoiesis in the presence of reduced amounts of Epo comprising co-administering a heme-containing composition and an Epo composition to erythroid cells wherein the amount of Epo administered produces an effective concentration of less than about 5 U/ml.

16. The method of claim 15 wherein the effective concentration is less than about 1 U/ml.

17. The method of claim 15 wherein co-administering involves administering the heme-containing composition and the Epo composition at about the same time.

18. The method of claim 15 wherein the erythroid cells comprises erythroid stem cells, erythroid progenitor cells, red blood cells or combinations thereof.

19. A method for the specific stimulation of erythropoiesis comprising administering a heme-containing composition to erythroid cells wherein said composition is substantially free of hemoglobin protein.

20. The method of claim 19 wherein the heme-containing component is hemin or hematin.

21. The method of claim 19 wherein erythropoiesis comprises proliferation and differentiation of CFU-GEMM cells, BFU-E cells or CFU-E cells.

22. The method of claim 19 wherein the specific stimulation of erythropoiesis occurs in the presence of a reduced concentration of Epo.

23. The method of claim 22 wherein the reduced concentration of Epo is a plasma concentration of less than about 10 mU/ml.

24. A composition comprising a heme-containing component coupled to an Epo-containing component.

25. The composition of claim 24 wherein the heme-containing component is heme, a hemin, a hematin, a hemoglobin or a fragment thereof.

26. The composition of claim 25 wherein the hemoglobin is a cross-linked hemoglobin, a recombinant hemoglobin or a chemically or genetically modified hemoglobin.

27. The composition of claim 24 wherein the Epo-containing component is Epo or an active portion thereof.

28. The composition of claim 24 wherein the heme-containing component is covalently coupled to the Epo-containing component.

29. The composition of claim 28 wherein the covalent coupling is via a peptide bond.

30. The composition of claim 28 wherein the covalent coupling is via an ionic bond.

31. A method for alleviating one or more symptoms associated with anemia comprising administering a composition comprising a heme-containing component to a patient wherein said composition is substantially free of hemoglobin protein.

32. The method of claim 31 wherein the anemia is selected from the group consisting of sickle cell anemias, thalassemias, infectious anemias, aplastic anemias, hypoplastic and hypoproliferative anemias, sideroblastic anemias, myelophthisic anemias, antibody-mediated anemias, anemias due to chronic disease and enzyme deficiency, and anemias due to blood loss, radiation therapy and chemotherapy.

33. A method for alleviating one or more symptoms of an anemia in a patient with a reduced endogenous level of Epo comprising administering a composition comprising a heme-containing component to said patient.

34. The method of claim 33 wherein the reduced endogenous level of Epo is less than about 10 mU of Epo per ml of plasma.

35. The method of claim 33 further comprising administering Epo to the patient wherein the amount administered is less than about 10 U per ml.

36. A method for promoting erythropoiesis *in vivo* comprising administering a composition comprising a heme-containing component and Epo to a patient wherein the Epo is administered to produce a blood plasma concentration of less than about 5.0 U/ml.

37. The method of claim 36 wherein the blood plasma concentration is less than about 1.0 U/ml.

38. The method of claim 36 wherein the composition is substantially free of hemoglobin protein.

39. The method of claim 36 wherein the heme-containing component is hemin or hematin.

40. A method for providing usable iron to an iron-deficient patient comprising administering a heme-containing component to the iron-deficient patient.

41. A method for treating porphyria comprising administering a heme-containing component to a patient.

42. The method of claim 41 wherein the porphyria is acute hepatic porphyria.

43. A method for differentiating erythroid cells comprising administering an effective amount of a heme-containing component to said erythroid cells.

44. The method of claim 43 wherein the erythroid cells are erythroid stem cells or erythroid progenitor cells.

45. The method of claim 43 wherein the heme-containing component is administered to said erythroid cells *in vitro* or *in vivo*.

46. A method for delivering heme to cells comprising administering a heme-containing component to said cells.

47. The method of claim 46 wherein the heme-containing component is administered to said cells *in vitro* or *in vivo*.

48. The method of claim 46 wherein the heme-containing component is hemin, hematin or hemoglobin.

49. A method for stimulating erythropoiesis in an Epo-deficient neonate comprising administering a heme-containing component to said neonate.

50. A method for transplanting stem cells comprising administering a heme-containing composition to a patient in association with transplanting stem cells.

51. The method of claim 50 wherein the composition is administered subsequent to transplanting.

52. The method of claim 50 wherein the composition is administered *ex vivo* prior to transplanting.

53. The method of claim 50 wherein the stem cells are obtained from bone marrow, cord blood, leukophoresis, adult peripheral blood or a combination thereof.

54. A method for accelerating red cell engraftment upon stem cell transplantation comprising administering a heme-containing component to a patient in association with said stem cell transplantation.

55. The method of claim 54 wherein the heme-containing component is administered *ex vivo* prior to transplantation.

56. A method for accelerating red cell engraftment comprising administering a heme-containing composition to a patient in association with said engraftment.

57. A method for reducing the toxicity of a chemotherapeutic agent administered to a patient comprising administering a heme-containing composition in associate with said chemotherapeutic agent to the patient.

58. The method of claim 57 wherein the chemotherapeutic agent is selected from the group consisting of acyclovir, ganciclovir, famciclovir, foscarnet, ribavirin, zalcitabine, azidothymidine, stavudine, lamivudine, didanosine, cytarabine, dideoxyadenosine, edoxudine, floxuridine, idozuridine, inosine pranobex, 2'-deoxy-5-(methylamino)uridine, trifluridine and vidarabine.

59. The method of claim 57 wherein the heme-containing composition comprises a component selected from the group consisting of heme, hemin, hematin, hemoglobin, purified hemoglobin, recombinant hemoglobin, cross-linked hemoglobin, modified hemoglobin and mutated hemoglobin protein.

60. The method of claim 57 wherein the patient is immunosuppressed.

61. The method of claim 60 wherein the immunosuppression is caused by an organ

transplantation, a viral infection or aquired immunodeficiency disorder.

62. The method of claim 61 wherein the viral infection is infection by Epstein Barr virus, adenovirus or cytomegalovirus.

63. A method for hemodilution comprising administering a heme-containing composition to a patient in association with said hemodilution.

64. The method of claim 63 further comprising administering Epo.

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FIGURE 1

Fold Expansion of Umbilical Cord Blood Progenitors at Ambient O_2

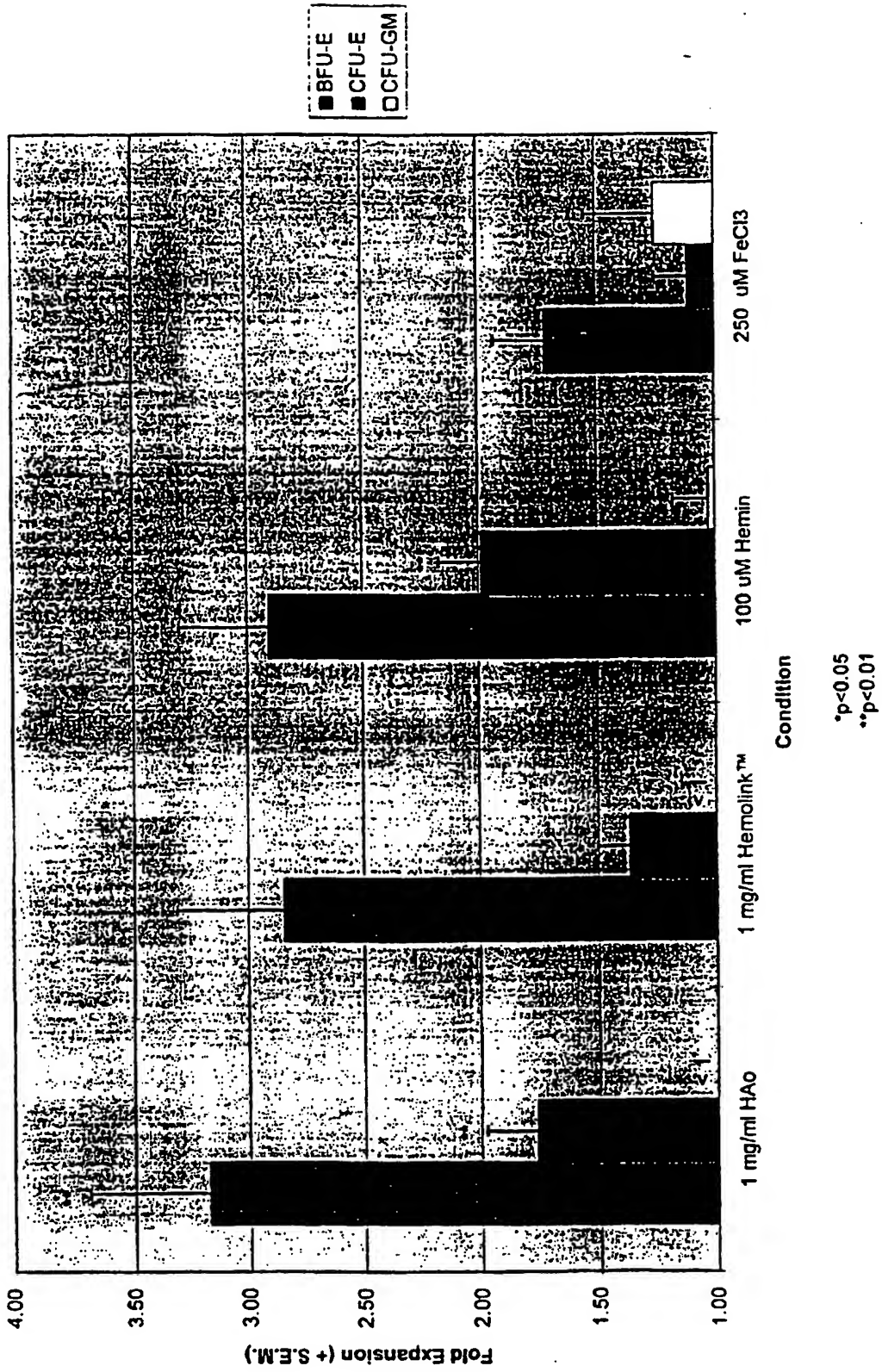


FIGURE 2

Fold Expansion of Umbilical Cord Blood Progenitors at 5% O_2

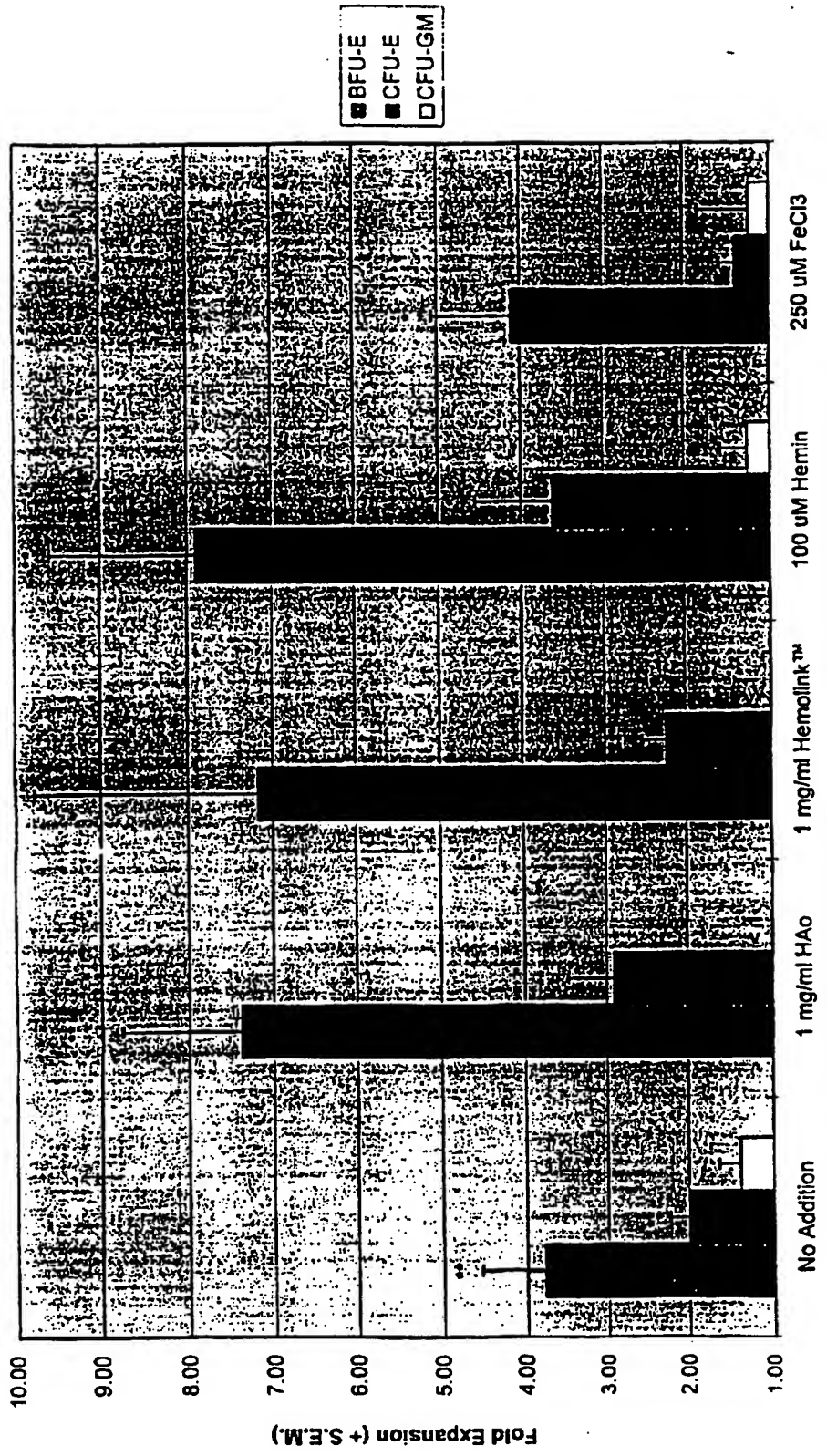


FIGURE 3

Fold Expansion of Umbilical Cord Blood Progenitors with 0.2 Units of Epo at Ambient O_2

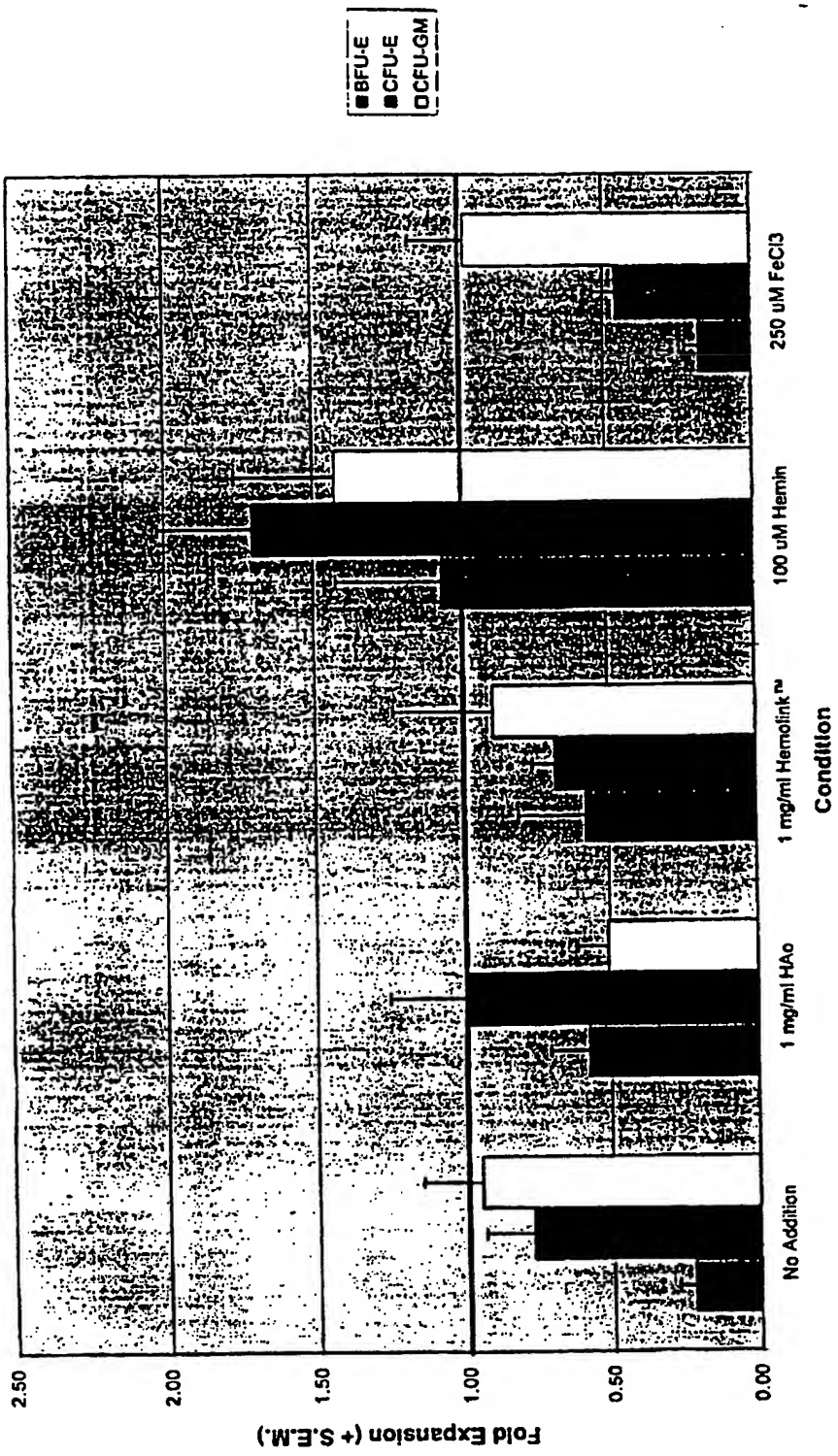


FIGURE 4

Fold Expansion of Umbilical Cord Blood Progenitors with 0.2 Units of Epo at 5% O_2

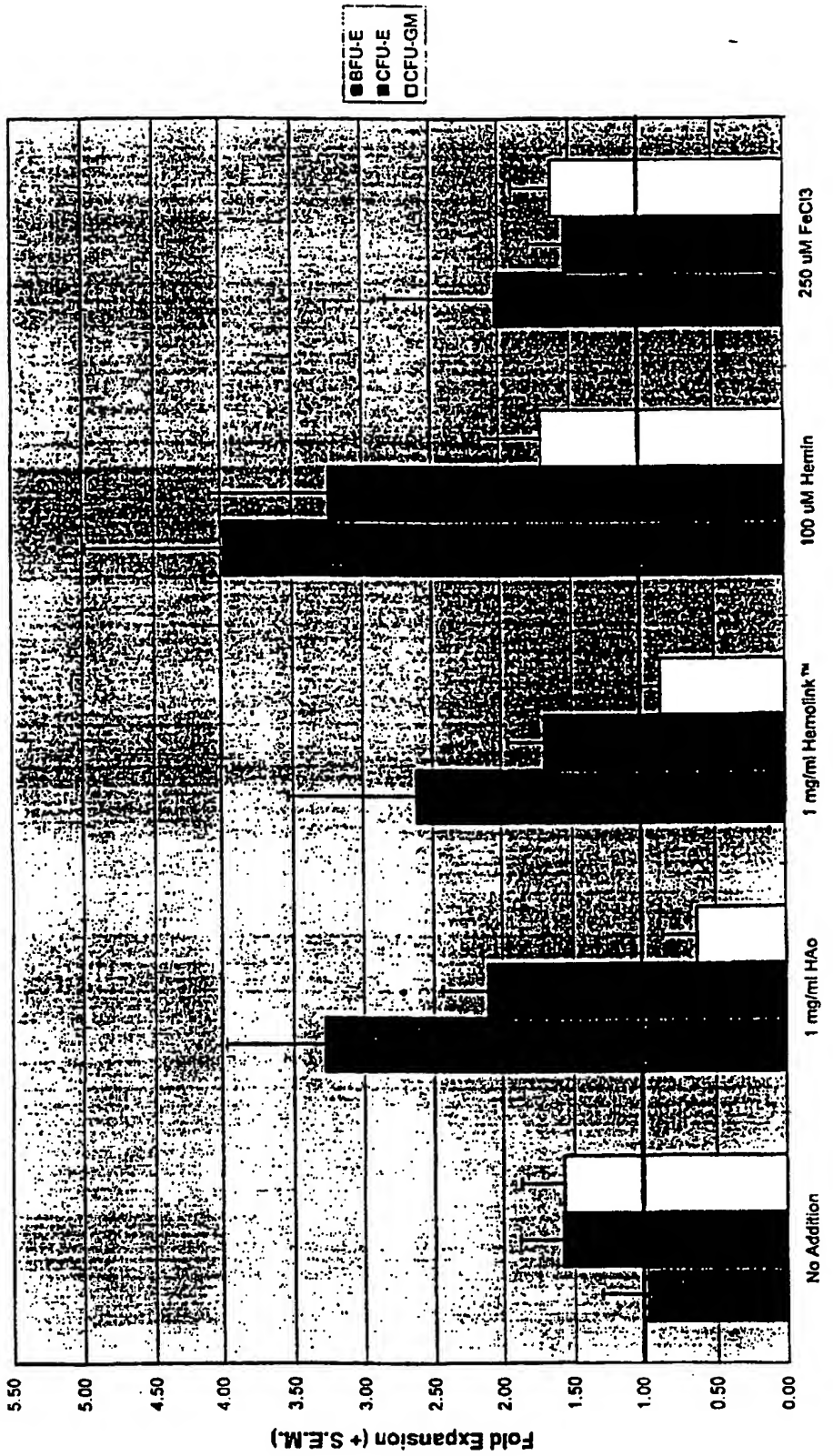
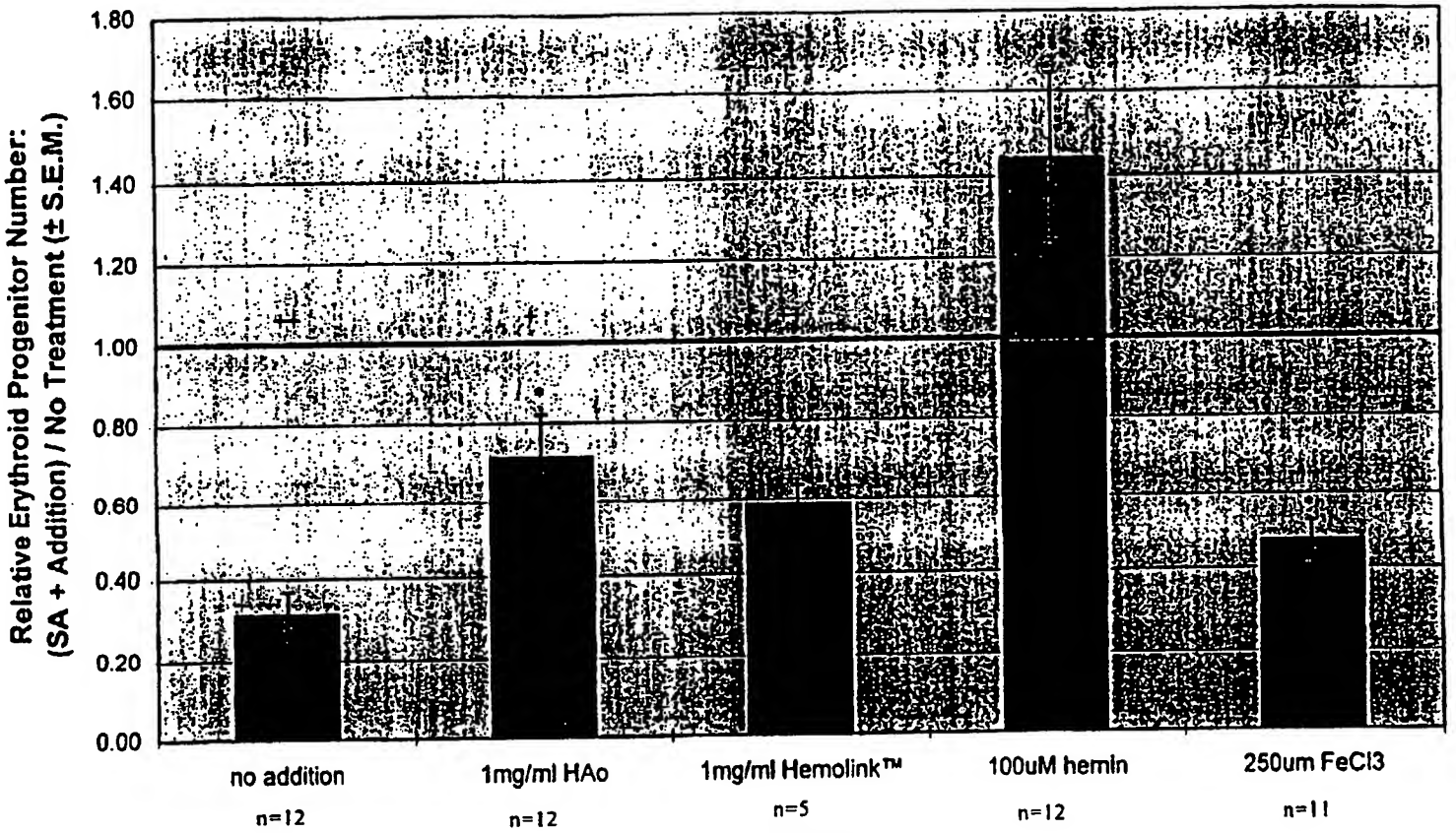


FIGURE 5

Inhibition of Succinylacetone (SA) Toxicity to Erythroid Progenitors



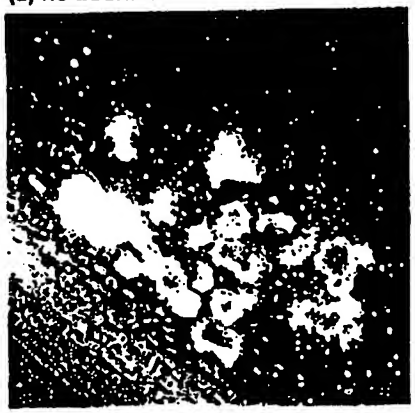
* $p < 0.001$ vs. no addition (> 0.31)
** $p < 0.0001$ vs. no addition (> 0.31)
+ $p < 0.05$ vs. no treatment (< 1.00)
++ $p < 0.001$ vs. no treatment (< 1.00)

Figure 6

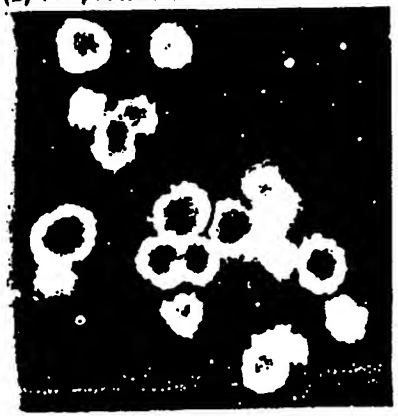
Representative Erythroid Colonies Which Form Under the Following Conditions:

2 Units/ml Epo; 5% O_2

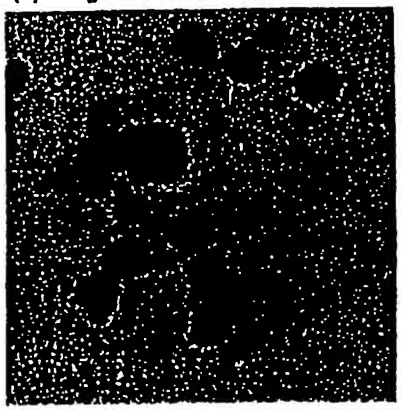
(a) no addition



(b) 100 μM hemin

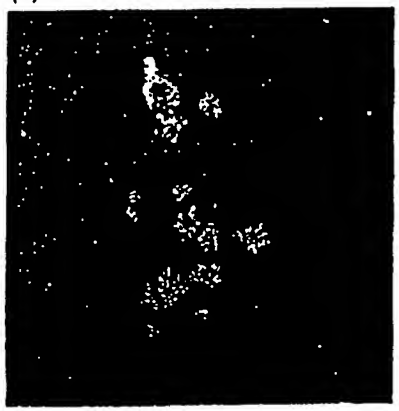


(c) 1 mg/ml HAo

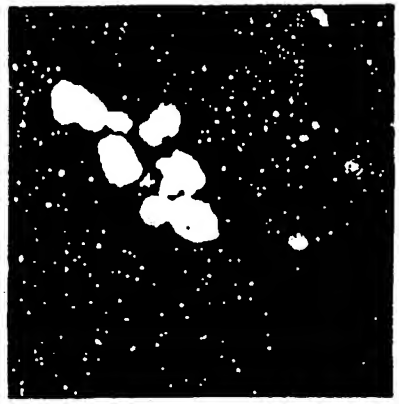


0.2 Units/ml Epo; 5% O_2

(d) no addition



(e) 100 μM hemin



(f) 1 mg/ml Hemolink™

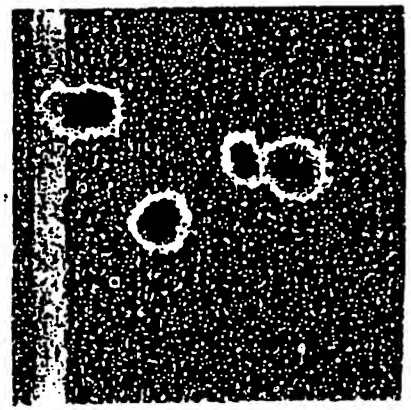


FIGURE 7

HPLC Analysis of Hemoglobin Production per CFA

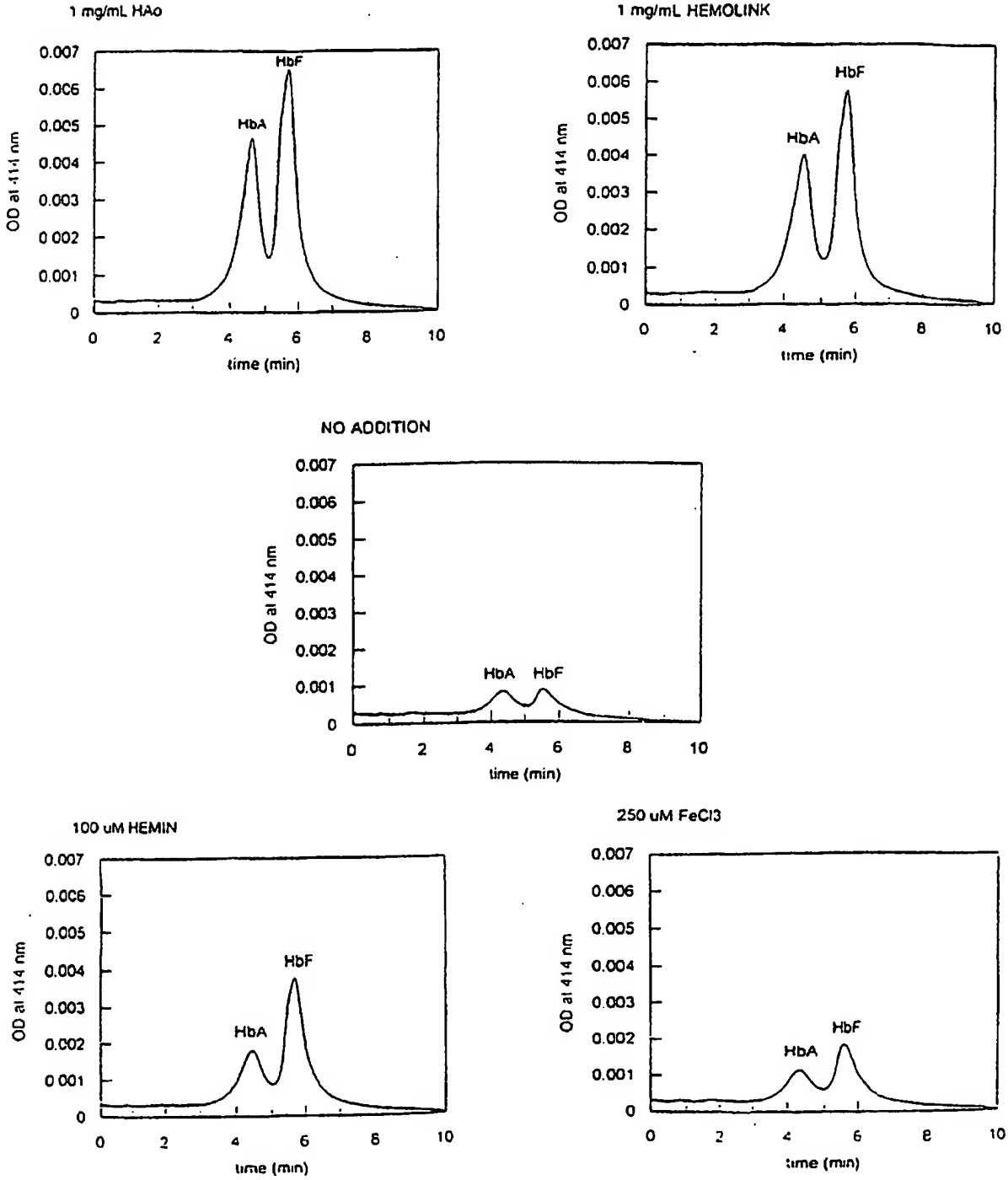
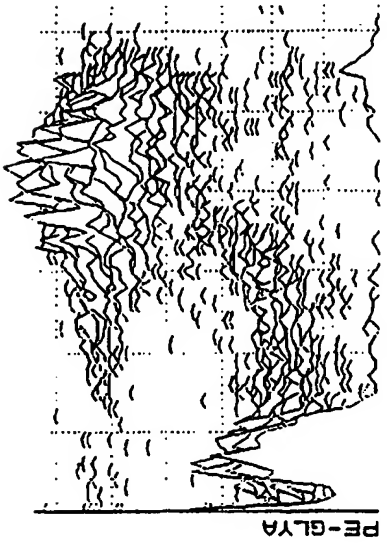


FIGURE 8

Purified Adult Hemoglobin Enhances Glycophorin A/CD71 Co-Expression in Erythroid Cells in Liquid Culture

(a) No Addition



(b) 1.0 mg/ml HAo

FIGURE 9

Fold Expansion of Adult Blood Progenitors at Ambient O_2

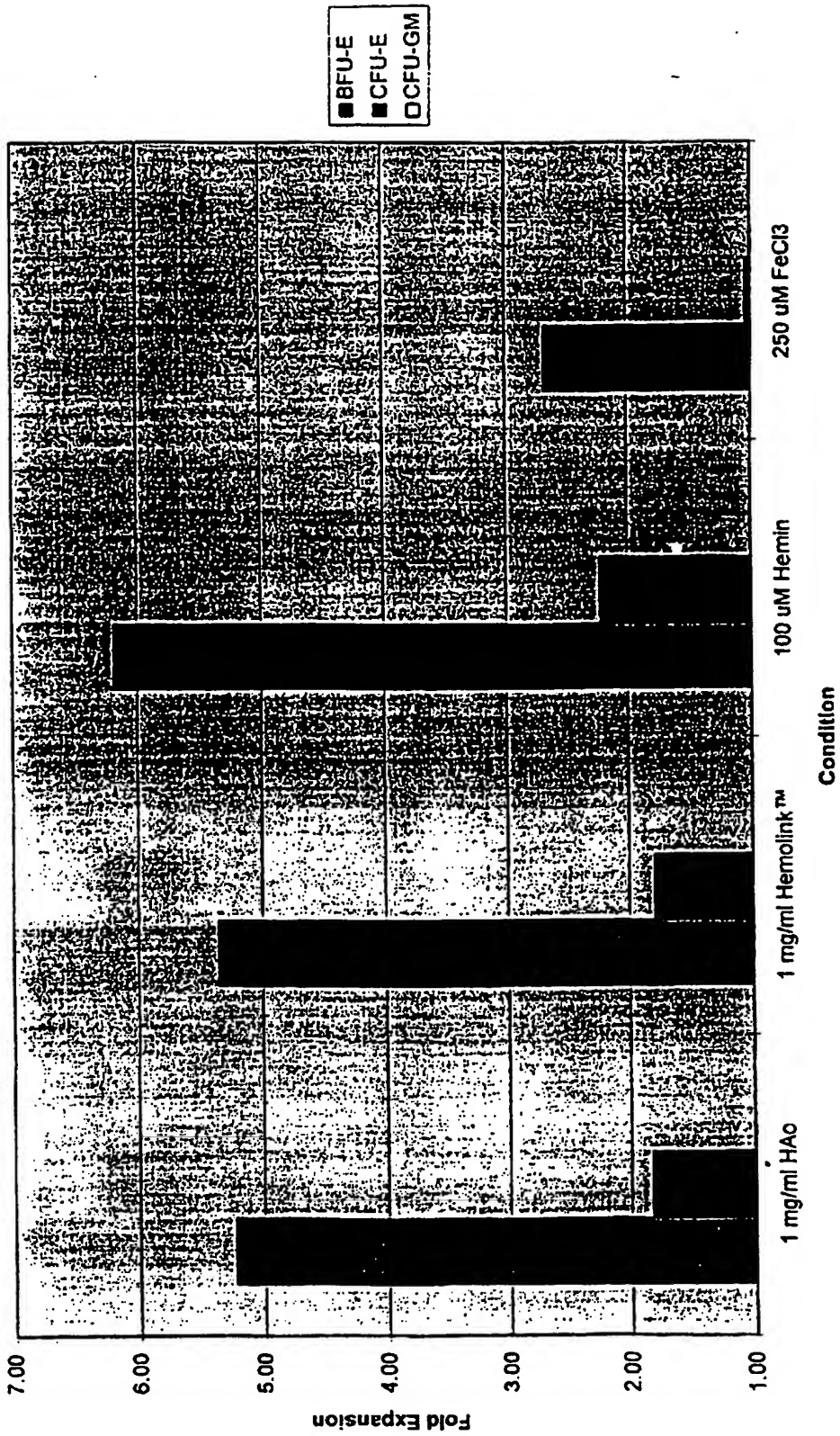


FIGURE 10

Fold Expansion of Adult Blood Progenitors at 5% O_2

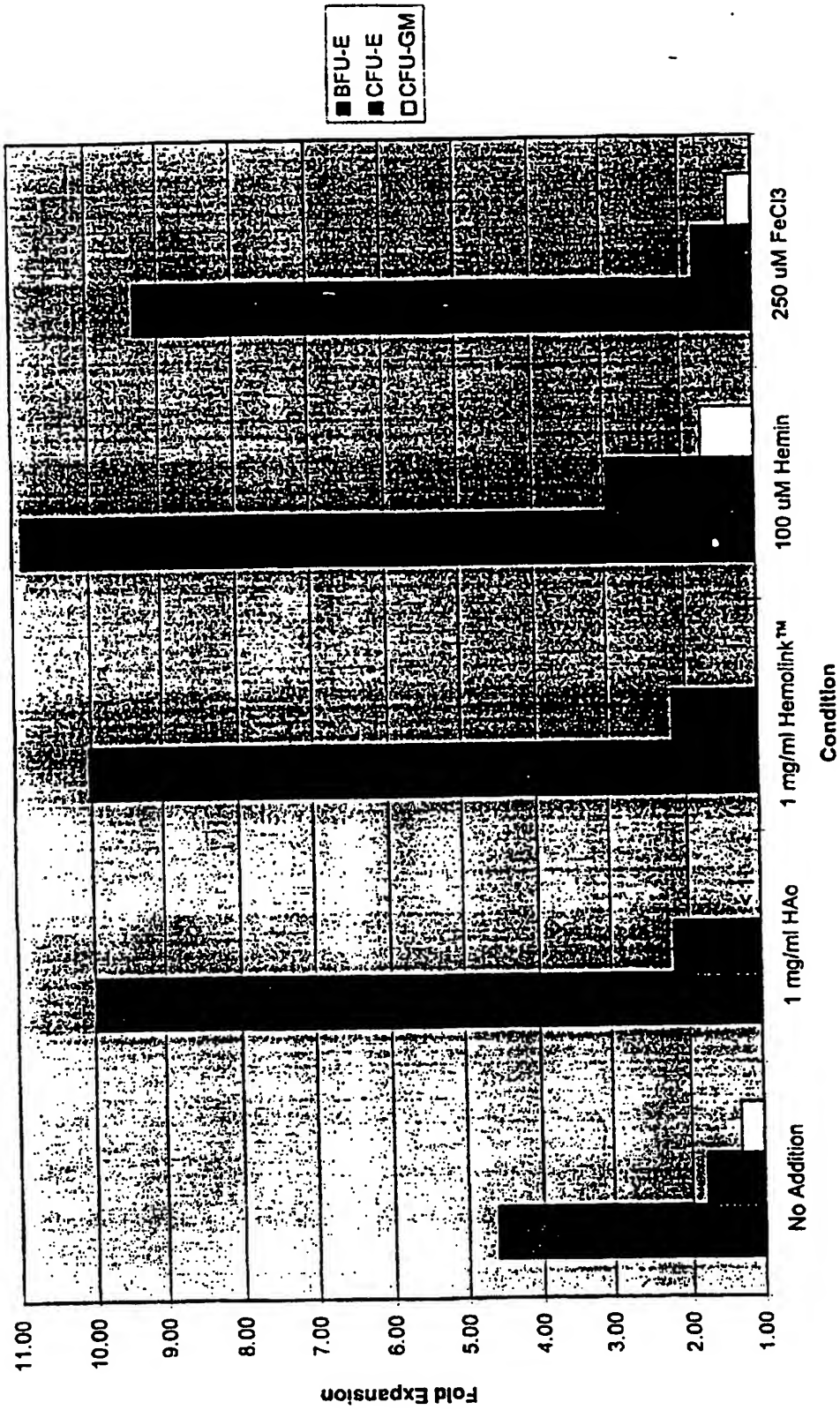


FIGURE 11

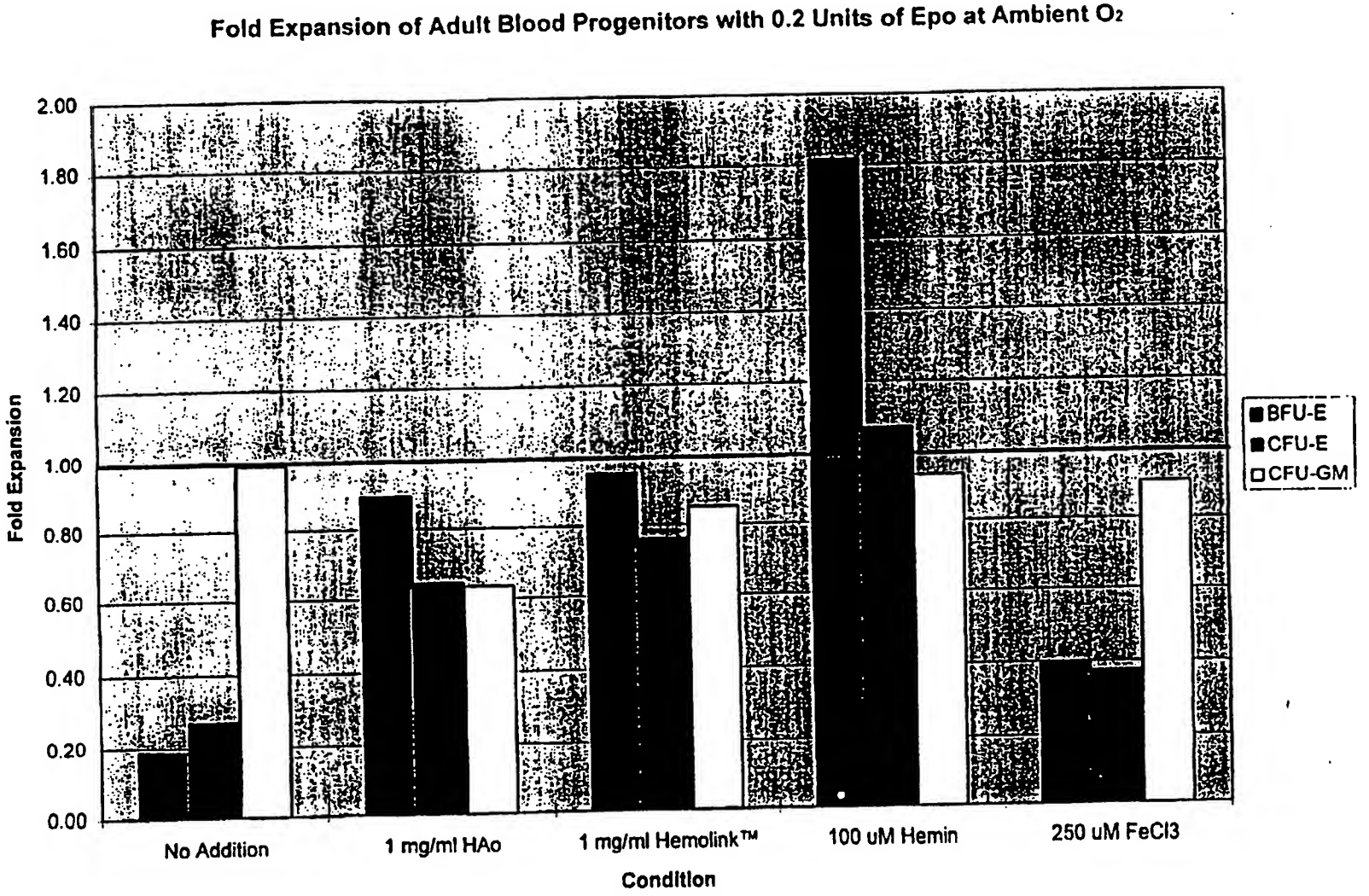


FIGURE 12

Fold Expansion of Adult Blood Progenitors with 0.2 Units of Epo at 5% O_2

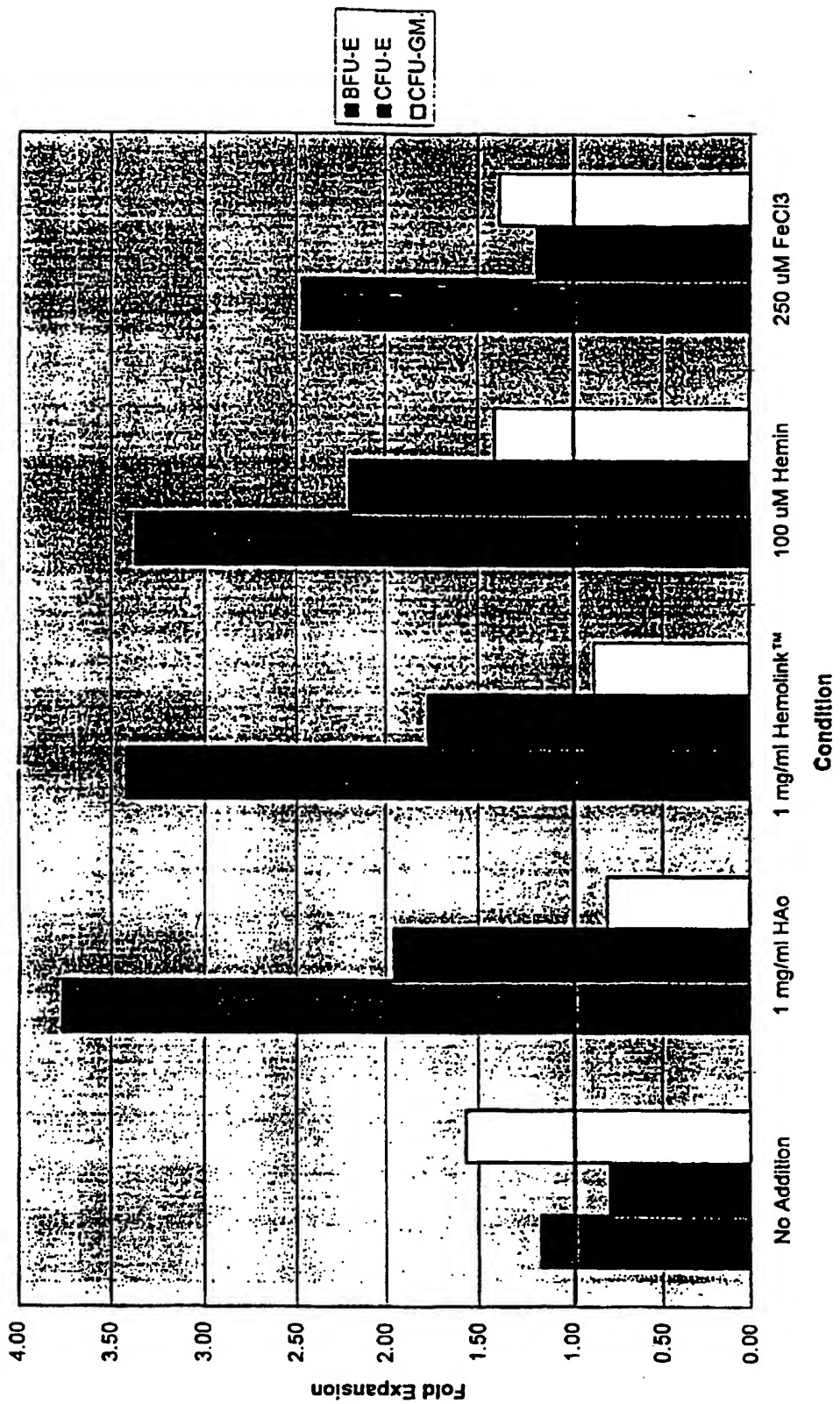
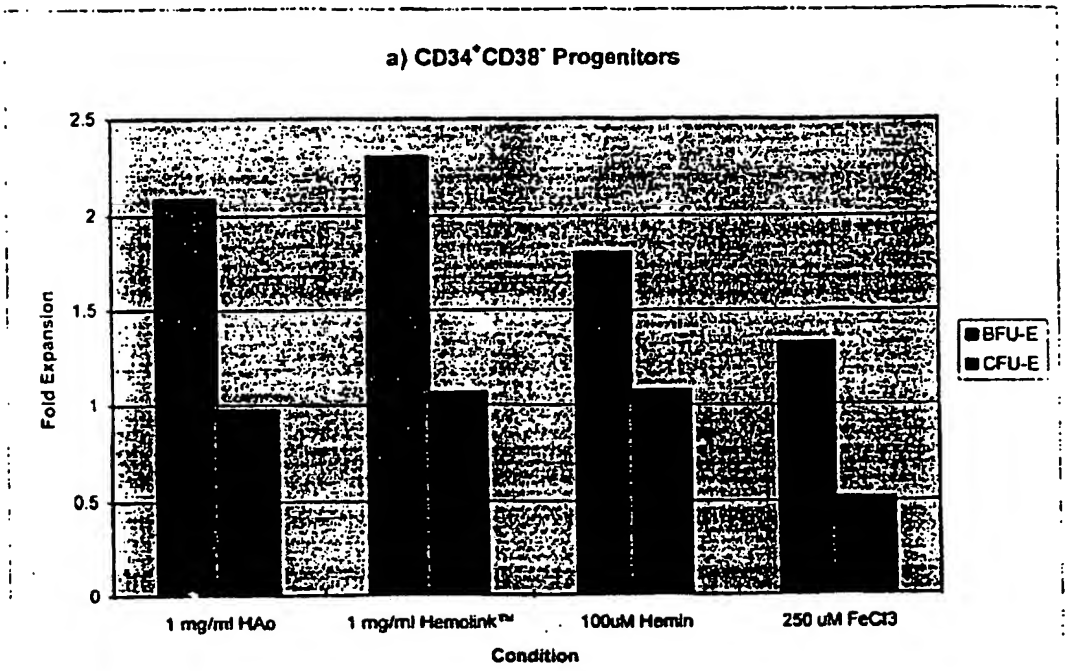


FIGURE 13

Fold Expansion of $CD34^+$ Progenitors with 2 Units of Epo at 5% O_2



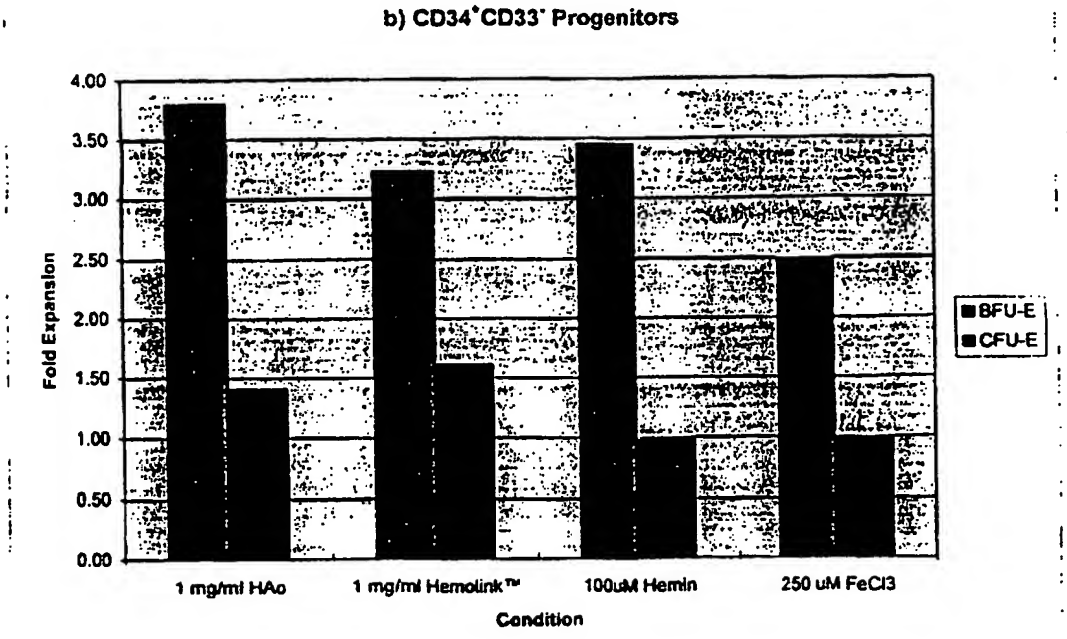


FIGURE 14

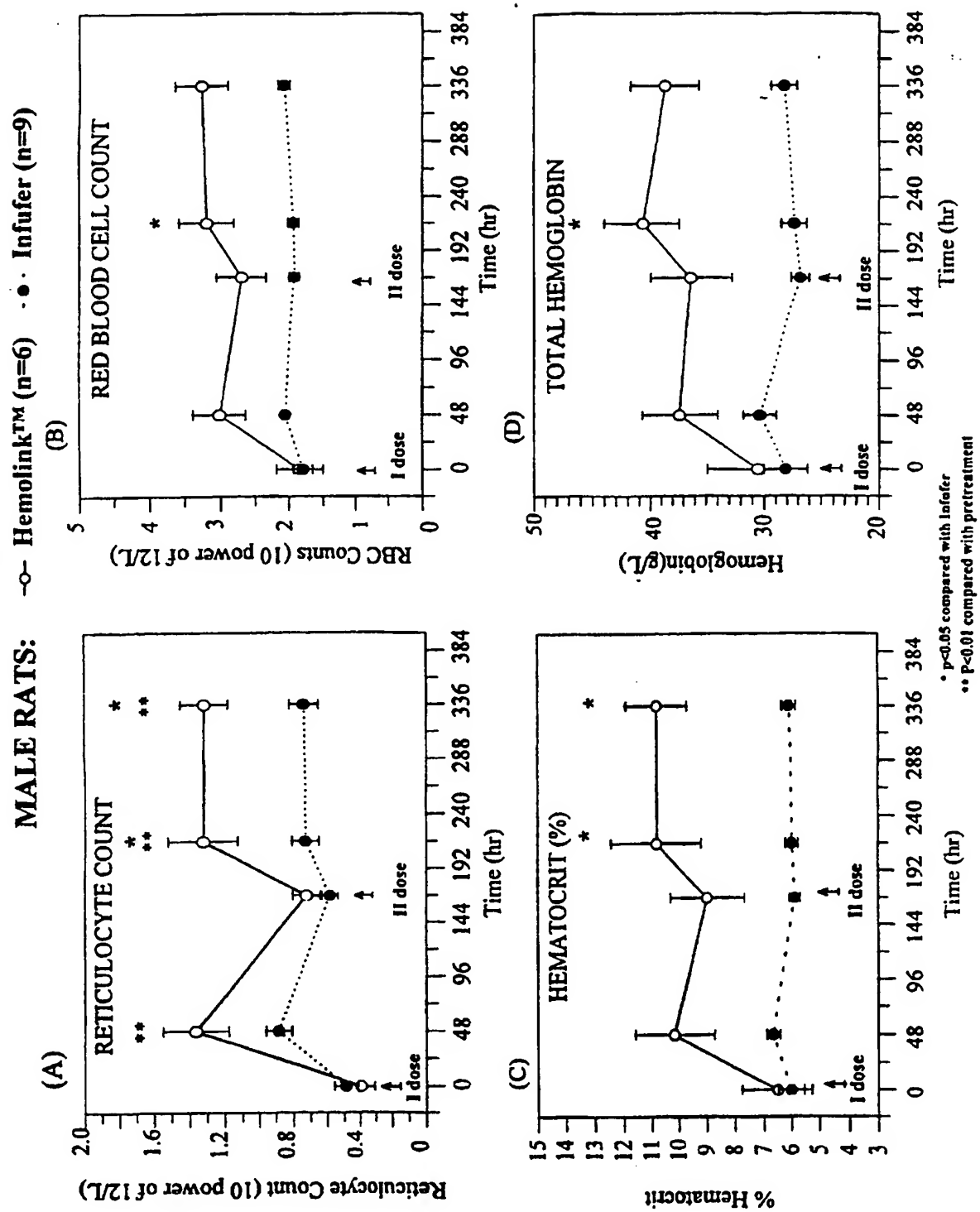
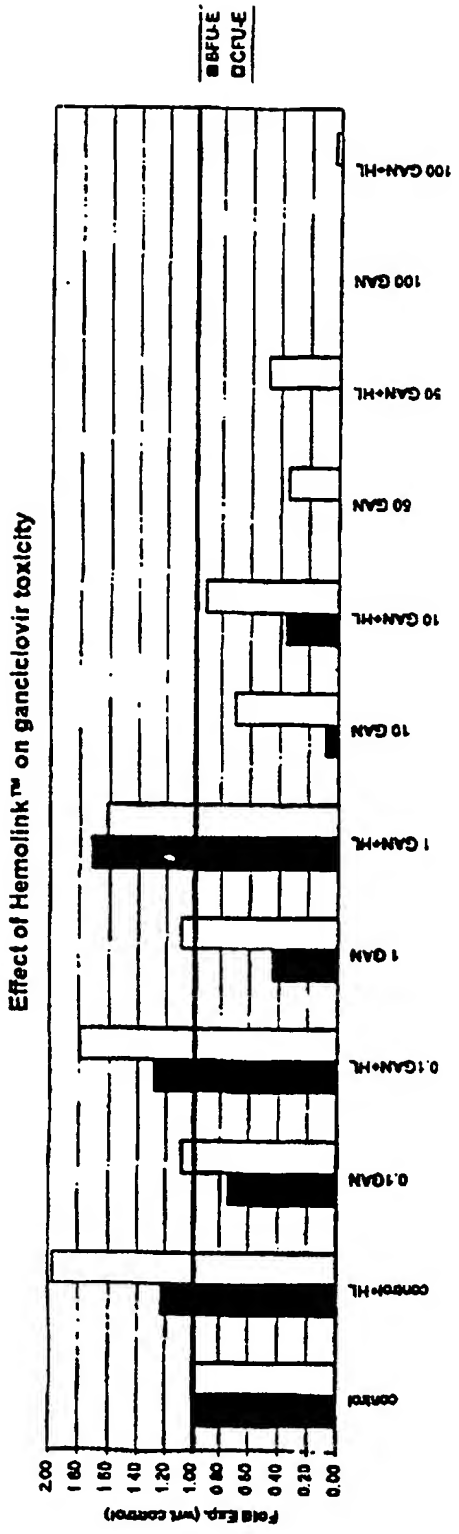

MALE RATS: -o- Hemolink™ (n=6) -•- Infufer (n=9)
(A)
RETICULOCYTE COUNT
Reticulocyte Count (10 power of 12/L)
(B)
RED BLOOD CELL COUNT
RBC Counts (10 power of 12/L)
(C)
HEMATOCRIT (%)
% Hematocrit
(D)
TOTAL HEMOGLOBIN
Hemoglobin(g/L)
Time (hr)
I dose
II dose
* p<0.05 compared with Infufer
** P<0.01 compared with pretreatment

FIGURE 15



Colony counts:

	control	control+HL	0.1GAN*	0.1GAN+HL	1 GAN	1 GAN+H	10 GAN	10 GAN+HL	50 GAN	50 GAN+HL	100 GAN	100 GAN+HL
BFU-E	70	86	52	80	31	120	8	25	0	0	0	0
CFU-E	222	438	241	399	242	359	156	203	77	108	0	8
CFU-GM	13	3	12	0	12	0	8	0	0	0	0	0

Fold Expansion:

	control	control+HL	0.1GAN	0.1GAN+HL	1 GAN	1 GAN+H	10 GAN	10 GAN+HL	50 GAN	50 GAN+HL	100 GAN	100 GAN+HL
BFU-E	1.00	1.23	0.74	1.29	0.44	1.71	0.09	0.36	0.00	0.00	0.00	0.00
CFU-E	1.00	1.97	1.09	1.80	1.09	1.62	0.70	0.81	0.35	0.49	0.00	0.04
CFU-GM	1.00	0.23	0.92	0.00	0.92	0.00	0.62	0.00	0.00	0.00	0.00	0.00

* 0.1GAN = 0.1uM ganaclovir

INTERNATIONAL SEARCH REPORT

Internatl Application No
PCT/CA 97/00601

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/42 A61K31/555 A61K38/18 A61K35/14 A61K35/28
A61K45/06 C12N5/06 C12N5/08 //(A61K38/42,38:18),
(A61K38/18,31:555)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. HARRISON ET AL.: "ADDITIVE EFFECT OF ERYTHROPOIETIN AND HEME ON MURINE HEMATOPOIETIC RECOVERY AFTER AZIDOTHYMIDINE TREATMENT." BLOOD, vol. 82, no. 12, 15 December 1993, NEW YORK, N.Y., US, pages 3574-3579, XP002049731 see page 3577, left-hand column, paragraph 2 - page 3578, left-hand column, paragraph 3; figures 3-5; table 1 ---	1-8, 13-25, 27-64
X	WO 92 02242 A (THE ROCKEFELLER UNIVERSITY) 20 February 1992 see page 6, line 20 - line 25; claims; figure 5 see page 13, line 11 - line 24 --- -/--	1-8, 13-25

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
10 December 1997	09.01.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ryckebosch, A

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INTERNATIONAL SEARCH REPORT

Internatio pplication No
PCT/CA 97/00601

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 24213 A (SOMATOGEN, INC.) 14 September 1995 see page 4, line 23 - page 5, line 7; claims 1-21,24-29; figures 1-8; examples see page 6, line 19 - line 23 see page 8, line 3 - page 9, line 17 see page 11, line 16 - line 18 see page 29, line 4 - line 34 see page 33, line 23 - page 35, line 14	-1-8, 11-19, 21-38, 40-64
X	WO 93 09220 A (P. CORREA ET AL.) 13 May 1993 see page 6, paragraph 3; claims 9,10,14 see page 14, paragraph 2	1-6
X	F. CORMIER ET AL.: "DÉVELOPPEMENT DES PROGÉNITEURS ÉRYTHROÏDES PRÉCOCES (BFUe) DE LA MOELLE OSSEUSE DE SOURIS DANS UN MILIEU DE CULTURE DÉPOURVU DE SÉRUM. EFFET DE L'HÉMINE." COMPTES RENDUS DES SEANCES DE L'ACADEMIE DES SCIENCES. SERIE III: SCIENCES DE LA VIE., vol. 299, no. 6, 30 July 1984, MONTREUIL FR, pages 143-146, XP002049732 see the whole document	1-6

1

INTERNATIONAL SEARCH REPORT

Inter. ..nal application No.
PCT/CA 97/00601

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 1-23 and 43-48 (all as far as being related to an in vivo method), 31-42, 49-64 are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internati Application No
PCT/CA 97/00601

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9202242 A	20-02-92	AU 645131 B	06-01-94
		AU 8410491 A	02-03-92
		CA 2088593 A	01-02-92
		EP 0543902 A	02-06-93
		JP 5504774 T	22-07-93
WO 9524213 A	14-09-95	US 5631219 A	20-05-97
		AU 2227095 A	25-09-95
		CA 2182800 A	14-09-95
		EP 0749318 A	27-12-96
		FI 963482 A	05-09-96
		HU 74846 A	28-02-97
		NO 963742 A	07-11-96
		PL 316145 A	23-12-96
WO 9309220 A	13-05-93	AU 2882992 A	07-06-93
		CA 2123094 A	13-05-93
		US 5397706 A	14-03-95